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Neuregulin-1 beta 1 is implicated in pathogenesis of multiple sclerosis

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Multiple sclerosis is characterized by immune mediated neurodegeneration that results in progressive, life-long neurological and cognitive impairments. Yet, the endogenous mechanisms underlying multiple sclerosis pathophysiology are not fully understood. Here, we provide compelling evidence that associates dysregulation of neuregulin-1 beta 1 (Nrg-1β1) with multiple sclerosis pathogenesis and progression. In the experimental autoimmune encephalomyelitis model of multiple sclerosis, we demonstrate that Nrg-1β1 levels are abated within spinal cord lesions and peripherally in the plasma and spleen during presymptomatic, onset and progressive course of the disease. We demonstrate that plasma levels of Nrg-1β1 are also significantly reduced in individuals with early multiple sclerosis and is positively associated with progression to relapsing-remitting multiple sclerosis. The functional impact of Nrg-1β1 downregulation preceded disease onset and progression, and its systemic restoration was sufficient to delay experimental autoimmune encephalomyelitis symptoms and alleviate disease burden. Intriguingly, Nrg-1β1 therapy exhibited a desirable and extended therapeutic time window of efficacy when administered prophylactically, symptomatically, acutely or chronically. Using *in vivo* and *in vitro* assessments, we identified that Nrg-1β1 treatment mediates its beneficial effects in EAE by providing a more balanced immune response. Mechanistically, Nrg-1β1 moderated monocyte infiltration at the blood-CNS interface by attenuating chondroitin sulphate proteoglycans and MMP9. Moreover, Nrg-1β1 fostered a regulatory and reparative phenotype in macrophages, T helper type 1 (Th1) cells and microglia in the spinal cord lesions of EAE mice. Taken together, our new findings in multiple sclerosis and experimental autoimmune encephalomyelitis have uncovered a novel regulatory role for Nrg-1β1 early in the disease course and suggest its potential as a specific therapeutic target to ameliorate disease progression and severity.

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Abbreviations: CIS = clinically isolated syndrome; CSPG = chondroitin sulphate proteoglycan; DMT = disease modifying treatment; dpi = days post induction; dpp = days post peak; EAE = experimental autoimmune encephalomyelitis; Nrg-1β1 = neuregulin-1 beta 1; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis

Introduction

Multiple sclerosis is a complex chronic immune-mediated condition of the CNS that manifests as demyelination with concomitant axonal and neuronal degeneration resulting in neurological impairment (Reich *et al.*, 2018; Faissner *et al.*, 2019). Approximately 85% of multiple sclerosis patients present with a relapsing-remitting course of the disease (RRMS), and most of these individuals advance to secondary progressive multiple sclerosis (SPMS) within 15–20 years of disease manifestation (Weinshenker *et al.*, 1989). Moreover, in multiple sclerosis disease course, clinically isolated syndrome (CIS) describes an individual who presents with a first episode of neurologic dysfunction characterized by demyelination or inflammation in the CNS consistent with an multiple sclerosis relapse (Miller *et al.*, 2012). When accompanied by brain lesions suggestive of multiple sclerosis on MRI, CIS indicates a high probability of a subsequent diagnosis of multiple sclerosis (Kuhle *et al.*, 2015). Current clinical assessment of multiple sclerosis lacks sensitivity for early diagnosis and prediction of disease progression because it most commonly relies on MRI to detect demyelinating plaques in the CNS in conjunction with clinical presentation. This limitation mainly reflects critical knowledge gaps in our understanding of the cellular and molecular mechanisms underpinning multiple sclerosis pathogenesis and progression. Uncovering these endogenous mechanisms would allow identification of disease markers for early diagnosis and treatment of progressive multiple sclerosis.

Multiple sclerosis pathogenesis is driven by activation, expansion and infiltration of leucocytes into the CNS tissue. Accumulation of these leucocytes in perivascular cuffs at the blood–CNS interface, and their cellular interactions with resident glia within the CNS orchestrate a neuroinflammatory response that leads to immune-mediated demyelination (Agrawal *et al.*, 2011; Dong and Yong, 2019). Intriguingly, while innate and adaptive immune cells promote a pro-inflammatory milieu causing neurodegeneration in multiple sclerosis, they also play a pivotal role in the resolution of immune-mediated attack and facilitate tissue repair (Rawji and Yong, 2013; Baaklini *et al.*, 2019; Yong *et al.*, 2019). This diverse role of activated leucocytes and microglia reflects their heterogeneity across a spectrum of pro-inflammatory to reparative phenotype. Accumulating evidence suggests that the net inflammatory balance of immune response is largely determined by the microenvironment (Baaklini *et al.*, 2019). Thus, it is critical to unravel endogenous mechanisms that regulate the phenotype of immune response during the onset, progression and reparative stages of multiple sclerosis. Identifying regulatory mechanisms implicated in early stage of multiple sclerosis pathogenesis would aid in early diagnosis, disease prevention and personalized therapeutic approaches.

Neuregulin-1 (NRG1) is a signalling protein that plays important roles in development and physiology of the peripheral and central nervous systems (Kataria *et al.*, 2019). NRG1 is conventionally known for its critical role in oligodendrocyte development and myelination. However, in recent years, NRG1 has emerged as a new immune modulator in traumatic and ischaemic CNS injuries (Xu *et al.*, 2006; Li *et al.*, 2009, 2015; Alizadeh *et al.*, 2017, 2018; Kataria *et al.*, 2018; Shahriary *et al.*, 2019). Neuregulin-1 beta 1 (Nrg-1β1) is a major NRG1 isoform in the CNS that contains the epidermal growth factor (EGF) like domain; the functional domain of all NRG1 isoforms (Mei and Xiong, 2008; Mei and Nave, 2014). In traumatic spinal cord injury and lysolecithin-induced focal demyelinating lesions of the spinal cord, we have recently shown that Nrg-1β1 is dysregulated in these lesions, and its availability promotes oligodendrogenesis and remyelination (Gauthier *et al.*, 2013; Kataria *et al.*, 2018). Moreover, we and others have shown that Nrg-1β1 attenuates astrocyte reactivity and the pro-inflammatory response of microglia in CNS injuries by blocking TLR/Myd88/NF-κB axis (Simmons *et al.*, 2016; Alizadeh *et al.*, 2017, 2018).

Efforts have also been made to identify the importance of NRG1 in multiple sclerosis. Earlier studies showed loss of NRG1 expression in multiple sclerosis active lesions (Viehover *et al.*, 2001), decreased expression in lysolecithin induced focal demyelinating lesions (Kataria *et al.*, 2018), reduced expression of one of its binding receptor ERBB4 in human blood mononuclear cells in RRMS patient samples (Tynyakov-Samra *et al.*, 2011), and an association of promoter polymorphisms in *NRG1* gene with SPMS and PPMS patients (Bahadori *et al.*, 2015). Moreover, administration of the NRG1 isoform glial growth factor 2 (GGF2) promoted remyelination in a chronic relapsing mouse model of EAE (Cannella *et al.*, 1998). Taken together, although early work studied NRG1 in multiple sclerosis, there exists a significant gap in our knowledge about its expression profile within the CNS and peripherally during the course of the disease, and its impact on pathogenesis, progression and recovery in autoimmune-mediated demyelination.

In this study, using the multiple sclerosis mouse model of experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis patient samples, we report for the first time that Nrg-1β1 protein expression was dysregulated in early phase of multiple sclerosis pathogenesis. In EAE mice, Nrg-1β1 was significantly reduced in the plasma and spleen early

at presymptomatic phase that also persisted during onset and progression of the disease. In relevance to multiple sclerosis, we also demonstrate that plasma levels of Nrg-1β1 were significantly reduced in CIS patients as compared to normal individuals. In the CNS, Nrg-1β1 expression was also severely depleted in active demyelinating lesions of multiple sclerosis and EAE. We also investigated the functional relevance of Nrg-1β1 dysregulation to EAE progression and found that restoring the deficient levels of Nrg-1β1 through systemic administration delayed disease symptoms and ameliorated neurological severity in EAE mice. Our extensive therapeutic studies indicate that Nrg-1β1 therapy offers an extended therapeutic time window, as it was effective when administrated prophylactically, symptomatically, acutely or chronically in EAE mice. Our immunohistological, cytometric, cytokine profiling and proteomics studies in EAE determined that availability of Nrg-1β1 promotes a comprehensive immune regulatory response by modulating the phenotype of microglia and myeloid cells and regulating several key mediators of EAE immunopathogenesis and neurodegeneration. Taken together, our new findings implicate Nrg-1β1 in early phase of multiple sclerosis pathogenesis and its importance in disease progression. Intriguingly, our preclinical studies also indicate the promise of Nrg-1β1 as a potential treatment strategy for multiple sclerosis.

Materials and methods

Study design

To evaluate the potential role of Nrg-1β1 in multiple sclerosis disease pathogenesis, we assessed protein levels of Nrg-1β1 in plasma, spleen and spinal cords of the EAE mouse model. These observations were corroborated with post-mortem multiple sclerosis brain tissue and plasma of cohort of multiple sclerosis patients. Further, we evaluated the therapeutic potential of recombinant human Nrg-1β1 (rhNrg-1β1) in the EAE mouse model. We used various therapeutic time window including treatment administration at the peak, onset, prophylactically and post-peak. Of note, a clinical grade of rhNrg-1β1 has received approval from Food and drug Administration (FDA) for phase II and III clinical trials for chronic heart failure, indicating its safety (https://clinicaltrials.gov/ct2/show/NCT03388593). To elucidate the underlying mechanisms, cytokine profiling, flow cytometry and proteomics were performed. Animals were randomly allocated to treatment groups. Observers were blinded to experimental groups during clinical score assessments. All the experimental procedures and assessments were performed in blinded manner. No animals or samples in any of the experiments were excluded from data analyses, unless specified otherwise.

Animal studies

All animal procedures and experimental protocols were approved by the Animal Ethics Care Committee of the University of Manitoba in accordance with the policies established in the guide for the care and use of experimental animals prepared by the Canadian Council of Animal Care. Mice were housed with a 12-h light/dark cycle in standard plastic cages at 22°C. Drinking water and pelleted food were given *ad libitum*. For *in vivo* EAE studies, 266 C57BL/6 female mice (8 weeks old, Supplementary Table 1) and for *in vitro* experiments, 10 C57BL/6 female mice (10 weeks old) and 25 C57BL/6 female pups (1–3 days old) were used. All animals were provided by Central Animal Facility, University of Manitoba, Canada.

EAE induction and treatments

C57BL/6 female mice (8 weeks old) were provided by the Central Animal Facility of University of Manitoba, Canada. Mice were immunized with myelin oligodendrocyte glycoprotein (MOG)35-55 as described in the Supplementary material. EAE mice were randomly assigned to experimental groups: vehicle and Nrg-1β1. Animals in Nrg-1β1 group received daily subcutaneous injections of rhNrg-1β1 peptide (~8 kDa) containing the bioactive epidermal growth factor (EGF)-like domain (Shenandoah Biotechnology) at indicated doses. Vehicle animals received equivalent volume of 0.1% bovine serum albumin (BSA) in saline. Treatments were administered daily under different paradigms: at the time of EAE induction (prophylactically), at the onset of EAE symptoms (clinical score of 0.5), at the peak of the disease (clinical score of 2.5–3) or in delayed fashion at 4 days after reaching the peak. EAE mice in transient therapeutic paradigm received treatments for 7 days starting at the peak of the disease.

Histology and immunofluorescence staining

At identified end points, deeply anaesthetized mice (isoflurane/propylene glycol; 40:60 v/v) were perfused with cold 0.1 M of phosphate-buffered saline (PBS) and 3.5% paraformaldehyde (PFA) for immunohistochemical analyses. The method is described in detail in the Supplementary material. A list of the antibodies used in this study is provided in Supplementary Table 2.

Detection of reactive oxygen species

Reactive oxygen species production was detected in EAE mice at end point with intraperitoneal injection of dihydroethidium (DHE, 10 mg/kg) (Molecular Probes, Invitrogen) as described previously (Choi *et al.*, 2015). The DHE is oxidized by reactive species within the cell, providing an index of the production of reactive species. Mice were euthanized 3 h after DHE injection and transcardially perfused as described above. Oxidized DHE signals were imaged after co-labelling with DAPI and immunofluorescence intensity was measured by ImageJ (NCBI) and expressed as fold change in mean grey value normalized to naïve mice.

Cell preparation and flow cytometry

Single-cell suspensions of peripheral blood and spinal cord were prepared and immunolabelled according to standard methods as described in the Supplementary material. Antibodies used for flow cytometry are provided in Supplementary Table 3.

Immunoblotting for CSPGs and lipid peroxidation

Mouse lumbar spinal cord tissue was homogenized in NP-40 lysis buffer containing protease inhibitor cocktail (Sigma). Slot blotting (as described in the Supplementary material) was performed to detect the expression of chondroitin sulphate

proteoglycans (CSPGs) and oxidized lipids with antibody against GAG portion of native CSPGs (clone CS-56, Sigma) and oxidized phospholipids (E06, Avanti, Millipore Sigma), respectively.

Gelatin gel zymography for MMP enzymatic assessment

Gelatin gel zymography was performed to assess enzymatic activity of MMP2 and MMP9 in the EAE spinal cord tissue, as described previously (Alizadeh *et al.*, 2017). The procedure is summarized in the Supplementary material.

Multiplex electrochemiluminescence cytokine assay

Levels of cytokines and chemokines in the spinal cord tissue of EAE mice were measured using the V-PLEX Mouse Cytokine 29-Plex Kit (Meso Scale Discovery) according to the manufacturer's instructions as described in the Supplementary material.

Enzyme linked immunosorbent assay for Nrg-1β1 detection

Mouse spinal cord tissue was homogenized in NP-40 lysis buffer containing protease inhibitor cocktail (Sigma). Mouse blood was collected with cardiac puncture in EDTA coated tubes. Samples were centrifuged at 2500 rpm for 25 min (4°C). Blood plasma was collected and stored as aliquots at −80°C until analysis. ELISA kit (DuoSet ELISA Development System; R&D Systems; DY377) was used to specifically detect Nrg-1β1 in blood plasma, spinal cord and spleen tissue lysates. Nrg-1β1 sandwich ELISA assay was performed according to the manufacturer's instructions, with standards (125–4000 pg/ml) and loading 25 µg of protein from each sample from spinal cord and spleen lysates. The Nrg-1β1 levels were calculated as picograms per microgram of tissue. For blood plasma analysis, direct ELISA was performed in the same manner, with the exception of omitting the first coating antibody. Fifty microlitres of plasma samples were used for assay and results were expressed as picograms per microgram of plasma.

Astrocytes, microglia and bone marrow derived macrophage *in vitro* studies

Astrocytes, microglia and bone marrow derived macrophage (BMDM) cultures were prepared as described in the Supplementary material. All three cells types were switched to serum free Dulbecco's modified Eagle media after 24 h of seeding and treated with vehicle (0.1% BSA), Nrg-1β1 (50 or 200 ng/ml), lipopolysaccharide (LPS) (100 ng/ml) + IFNγ (20 ng/ml) or Nrg-1β1 + LPS + IFNγ for 72 h. Conditioned media was collected and stored at −80°C until further use.

T helper 1 and T helper 17 polarization *in vitro*

Naïve CD4+ T cells were purified from the spleens and lymph nodes of 10-week-old female C57BL/6 mice using an EasySep™ Mouse Naïve CD4+ T Cell Kit (19765, Stemcell Technologies) Isolated Naïve $CD4^+$ T cells were cultured and polarized to T helper (Th)1 or Th17 conditions as described in the Supplementary material.

Proteomics procedures and analyses

LC-MS/MS sample preparation and analysis

Spinal cord lysates were digested, labelled and analysed by Manitoba Centre for Proteomics and Systems Biology (University of Manitoba) as per their standard procedures. Protein digests were performed as specified in the manufacturer's instructions for the Thermo Scientific's TMT10plex Isobaric Mass Tagging Kit (Cat. #90110). TMT labelling was performed according to the manufacturer's instructions to label each biological replicate with a unique tag.

Database for annotation, visualization, and integrated discovery analysis

The database for annotation, visualization, and integrated discovery (DAVID) (https://david.ncifcrf.gov/) v6.8 is a comprehensive tool to perform functional annotation and understand biological meaning behind large list of genes associated with proteins. We performed a gene ontology (GO) term enrichment analysis using DAVID and identified enriched biological themes and most relevant GO terms associated with our study. Only GO terms with an adjusted P-value < 0.05 were considered significant.

ClueGO analysis

ClueGO plug-in of Cytoscape (http://www.cytoscape.org/) was used to generate protein pathways and to constitute the network of pathways based on the Gene Ontology. ClueGO enables to visualize the non-redundant biological terms for large clusters of genes in a functionally grouped network (Bindea *et al.*, 2009). The parameters used for ClueGO analysis are described in the Supplementary material.

Human brain multiple sclerosis specimens

Frozen post-mortem brain tissues were obtained from the United Kingdom Multiple Sclerosis Tissue Bank at Imperial College, London (www.ukmstissuebank.imperial.ac.uk; provided by Richard Reynolds and Djordje Gveric). All multiple sclerosis tissues were obtained and used with approval from the institutional ethics committee of the University of Calgary. Six multiple sclerosis brain tissues with active lesions from individuals with chronic multiple sclerosis were assessed in this study. The lesions fulfilled the morphological criteria of an active inflammatory demyelinating process consistent with multiple sclerosis when stained with haematoxylin and eosin-Luxol fast blue. The details of patient samples are provided in Supplementary Table 4. Human tissue sections were fixed with 3.5% PFA before immunohistochemical staining as described in the Supplementary material.

Human plasma samples

Patients with multiple sclerosis and normal participants were recruited at the Winnipeg Health Sciences Center, Winnipeg, Canada. All patients were diagnosed with multiple sclerosis according to the 2010 revised McDonald criteria. Based on the clinical diagnosis, plasma samples were categorized into different types/stages of multiple sclerosis: CIS, RRMS and SPMS. Demographic and clinical characteristics of patients with multiple sclerosis and normal controls are described in

Supplementary Table 5. Healthy individuals served as controls. The study was approved by the Health Research Ethics Board of the University of Manitoba. All participants gave written informed consent. Human blood was collected in sodium heparin tubes by standard venepuncture procedure. For blood plasma analysis, direct ELISA was performed in the same manner as described above for mouse plasma samples. All the stratifications undertaken with human plasma samples are representative of *post hoc* analyses as these samples were repurposed from another unrelated clinical research project.

Statistical analysis

In all analyses, we performed unbiased assessments by utilizing randomization and blinding of methods. Using SigmaStat Software, Mann-Whitney U-test was used for human plasma data analysis. One-way ANOVA followed by Holm-Sidak *post hoc* correction was used when comparing more than two groups. Two-way ANOVA was used for analysis of neurological scoring in EAE studies while Holm-Sidak *post hoc* analyses was used when comparing mean of each time point. Mann-Whitney test was used while analysing EAE-based non-parametric data. Student's *t*-test was used when two groups were compared in EAE data. Specific statistical tests used for data analysis are described in respective figure legends. The data are reported as means ± standard error of the mean (SEM) unless specified otherwise and $P \leqslant 0.05$ was considered statistically significant in all the analyses.

Data availability

The data that support the findings of the study are available from the corresponding author upon reasonable request.

Results

Dysregulated levels of Nrg-1β1 protein is detected in EAE mice and precedes disease onset

We conducted an in-depth investigation on Nrg-1β1 protein expression pattern in the spinal cord and peripherally in plasma and spleen of MOG35-55 induced EAE mice. Immunohistological characterization of spinal cord lesions in EAE mice confirmed that Nrg-1β1 expression was significantly diminished within EAE demyelinating lesions (Fig. 1A–C). Nrg-1β1 immunofluorescence intensity measurement within the EAE lesions showed 48% reduction in Nrg-1β1 levels as compared to normal-appearing white matter and naïve spinal cord tissue sections (Fig. 1C). These findings provide the first evidence that dysregulation of Nrg-1β1 protein is a characteristic of EAE lesions of the spinal cord. Since NRG1 is primarily expressed by neurons/axons and oligodendrocytes, and to a lesser extent by astrocytes (Tokita *et al.*, 2001; Gauthier *et al.*, 2013; Kataria *et al.*, 2019), we asked whether decline in Nrg-1β1 within EAE lesions of white matter is attributed to loss of these cell types as the result of EAE. Our quantitative immunohistological assessment revealed 75% and 63% reduction in axonal and oligodendrocyte cell density, respectively, in EAE lesions as compared to naïve tissue and adjacent normal-appearing white matter area in the same EAE mouse (Supplementary Fig. 1A, B, D and E). On the contrary, immunofluorescence intensity measurement for astrocyte marker GFAP was significantly increased (60%) within the EAE lesions compared to naïve tissue and adjacent normal-appearing white matter area in the same EAE mouse (Supplementary Fig. 1C and F). Thus, these data suggest a positive correlation between diminished levels of Nrg-1β1 and reduced axonal and oligodendrocyte cell densities within the EAE lesions.

We also conducted a time point ELISA analysis for Nrg-1β1 protein levels in the spinal cord of EAE mice and found a significant downregulation (22%) at the presymptomatic phase [7 days post induction (dpi)], as compared to the baseline of Nrg-1β1 expression in the spinal cord of normal non-EAE mice (Fig. 1D and Supplementary Fig. 2A). The decline in Nrg-1β1 protein levels persisted at the onset (12 dpi, 22%), peak of the disease (16 dpi, 25%), 14 days post peak (dpp) (23%) that lasted chronically until 28 dpp (20%). However, at 7 dpp, there was a modest recovery in Nrg-1β1 levels by 18% as compared to the EAE peak, which could be possibly due to stochastic variations in samples. We further determined whether there is a correlation between peripheral levels of Nrg-1β1 in plasma and spleen with disease onset and progression. Interestingly, Nrg-1β1 level was significantly reduced in both plasma and spleen of EAE mice during the disease course at presymptomatic (7 dpi), onset (10–12 dpi) and peak (14–16 dpi), as compared to naïve animals (Fig. 1E and F). In plasma, Nrg-1β1 was significantly declined at 7 dpi (40%), onset (73%) and peak (50%) of EAE (Fig. 1E and Supplementary Fig. 2B). In spleen tissue, the magnitude of Nrg-1β1 depletion was even more pronounced, as it was barely detectable at presymptomatic, onset and peak of EAE as compared to naïve mice (Fig. 1F and Supplementary Fig. 2C). Nrg-1β1 levels were moderately recovered in plasma and spleen starting from 7 dpp until 28 dpp, the last time point of our analyses (Fig. 1E and F). To confirm the overall integrity of protein in EAE plasma and tissue samples, we used serum albumin (BSA) and GAPDH as housekeeping proteins, respectively. ELISA assessment showed no significant change the levels of serum albumin in the plasma or GAPDH in spleen and spinal cord lysates of EAE samples across various time points as compared to naïve samples (Supplementary Fig. 2D and F), confirming that changes in Nrg-1β1 protein expression is a biological event related to EAE pathology. Collectively, these findings underscore a strong correlation between EAE pathogenesis and progression and Nrg-1β1 downregulation within the CNS and peripherally in plasma and spleen.

Nrg-1β1 treatment reduces disease severity in EAE mice with an extended therapeutic time window

We next sought to determine whether CNS and systemic downregulation of Nrg-1β1 in EAE may have any functional

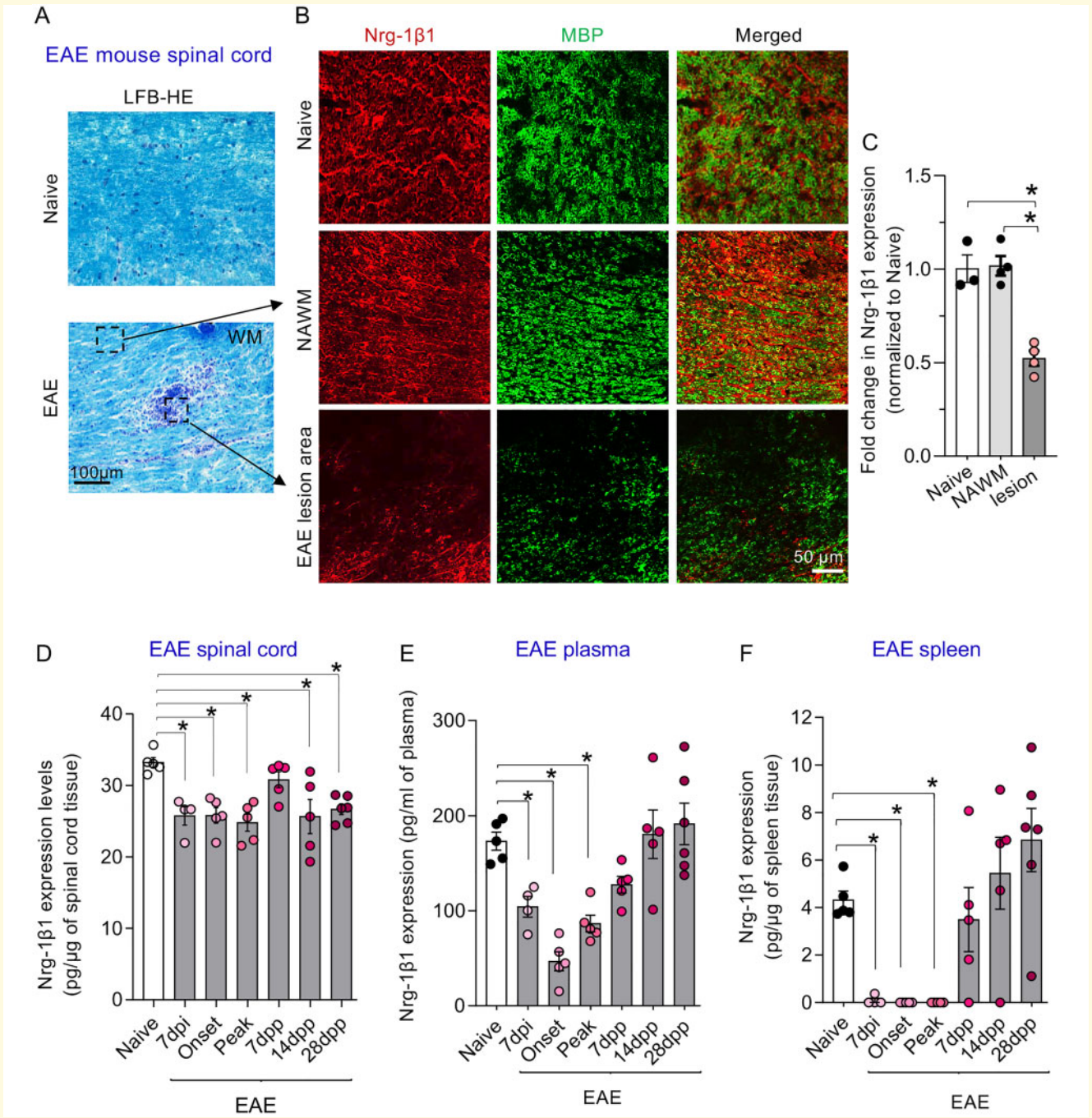

Figure 1 Nrg-1β1 expression levels are declined in CNS and peripherally in EAE mice. (**A**) Representative Luxol fast blue-haematoxylin and eosin (LFB-HE) stained spinal cord tissue from naïve and EAE mice at the peak of the disease indicating demyelinating lesions. (**B**) Immunohistological examination for myelin (MBP) and Nrg-1β1 revealed that Nrg-1β1 expression is depleted in demyelinated regions whereas adjacent myelinated normal appearing white matter area (normal-appearing white matter) as well as naive mice tissue indicated a strong expression of MBP and Nrg-1β1. (**C**) Quantitative immunofluorescence intensity in EAE spinal cord lesions showed 48% reduction in Nrg-1β1 as compared to the adjacent normal-appearing white matter and naïve mice tissue. Values are represented as fold change in intensity normalized to naïve. (**D**–**F**) Longitudinal assessment of Nrg-1β1 levels was performed on spinal cord (**D**), plasma (**E**) and spleen (**F**) of EAE mice at 7 dpi, onset (10 dpi), peak (14–16 dpi), 7 dpp, 14 dpp and 28 dpp. Nrg-1β1 was significantly depleted in plasma, spleen and spinal cord of EAE mice at 7 dpi, onset and peak of the disease. It was restored in plasma and spleen during the recovery phase (7 dpp, 14 dpp and 28 dpp). However, in the spinal cord there was another reduction Nrg-1β1 levels at 14 dpp in which persisted until 28 dpp. Values represent mean ± SEM. *$P < 0.05$; One-way ANOVA followed by Holm-Sidak *post hoc* test. $n = 3$–5. Naïve mean values were compared to each time point for *post hoc* test in **D**–**F.**

ramifications on disease progression and severity. To this end, we systemically administered human recombinant Nrg-1β1 to EAE mice through daily subcutaneous injections. We performed systemic intervention as a clinically relevant strategy and the notion that Nrg-1β1 was declined both peripherally and in the spinal cord. Of note, Nrg-1β1 is an ~8 kDa peptide containing the bioactive EGF-like domain that is essential for activation of NRG1 signalling. Importantly, previous pharmacokinetic studies with similar peptide (8 kDa) confirmed that Nrg-1β1 peptide can readily pass the blood–CNS barrier by saturable, receptor-mediated transport and enter CNS tissue (Kastin *et al.*, 2004). We first performed a dose efficacy study with different concentrations of Nrg-1β1 peptide delivered at 300 ng, 600 ng and 1200 ng per day. To simulate the common clinical management of multiple sclerosis, we began Nrg-1β1 therapy once an EAE mouse reached peak of the disease (around Day 14–16 post EAE induction, clinical score of 2.5–3 on a 5-point scale). EAE animals received daily treatment until 42 dpi. The control group received 0.1% BSA in saline, vehicle for Nrg-1β1, in the same manner. Daily clinical assessments by two experimenters blinded to animal treatments showed improved functional recovery in Nrg-1β1 treated EAE mice in a dose-dependent manner (Fig. 2A). At the end point (42 dpi), the lower 300 ng/day dose did not induce any beneficial effects on disability score compared to vehicle treated EAE mice, suggesting that this dose did not reach the therapeutic threshold of Nrg-1β1 peptide that is required to induce significant improvements in EAE clinical scores. At the same time point, we found that both 600 ng and 1200 ng daily dose of Nrg-1β1 significantly and comparably improved functional recovery (26%), suggesting the ceiling effect was reached with 600 ng dose (Fig. 2A). Of note, previous pharmacokinetic studies by Kastin *et al.* (2004) has shown that radioactively labelled Nrg-1-β1 peptide is relatively stable in mouse for 10 min after intravenous injection, and can cross the blood–CNS barrier by saturable, receptor-mediated transport and enters the parenchyma of brain and spinal cord (Kastin *et al.*, 2004). Thus, it is plausible that beyond certain dose (e.g. 600 ng used in our study), the receptor-mediated transport for Nrg-1β1 becomes saturated and the therapeutic efficacy of the peptide reaches its peak with no further improvement in neurological scores. Based on the daily clinical scores, we also calculated the area under the curve as representative of cumulative disease burden for each animal. Similarly, our analysis showed a significant reduction in cumulative disease burden in EAE animals that received 600 ng/day (21%) and 1200 ng/day (26%) of Nrg-1β1 as compared to the vehicle treated EAE mice (Fig. 2B). Moreover, a heat map plot for the clinical score of each individual mouse at the end point for vehicle and NRG1 (600 ng/day) treated group showed a significantly reduced disability score in Nrg-1β1 treated EAE animals compared to vehicle treated EAE mice (Fig. 2C). Based on this dosing study, we used 600 ng/day (30 μg/kg/day) as an effective dose for Nrg-1β1 in all subsequent EAE studies.

Since our findings showed Nrg-1β1 levels are markedly reduced early in EAE development, we asked whether restoration of Nrg-1β1 would ameliorate EAE severity and progression when treatment is administered at the onset of the EAE symptoms (Day 12) or prophylactically at the time of EAE induction. Our long-term longitudinal evaluation for 36 dpi showed Nrg-1β1 treatment starting at the EAE clinical onset significantly reduced the cumulative burden of disease (21%) when compared to vehicle treatment (Fig. 2D–F). Prophylactic Nrg-1β1 treatment at the time of EAE induction more pronouncedly improved neurological disability (55% reduction) and cumulative disease burden (53%) in EAE mice and also delayed EAE progression (Fig. 2G–I). Collectively, these findings suggest that dysregulation of Nrg-1β1 has functional ramifications in the pathogenesis of EAE.

We extended our therapeutic studies to assess whether Nrg-1β1 therapy would be therapeutically beneficial if administrated in a delayed fashion after the peak of EAE. Interestingly, Nrg-1β1 therapy also attenuated the severity of EAE disability (21%) when it was delayed to 4 dpp compared to the clinical disability of vehicle treated animals at the end point (Fig. 2J–L). We further evaluated the necessity of sustained administration of Nrg-1β1 therapy for EAE recovery. To this end, we administered Nrg-1β1 treatment transiently for 7 days, beginning at peak of the disease and assessed neurological recovery of EAE animals until 42 dpi. Interestingly, 7-day short-term treatment of Nrg-1β1 did not improve EAE-induced neurological deficits (Fig. 2M–O). Taken together, these observations suggest that dysregulation of Nrg-1β1 has significant implications in EAE onset and progression. Importantly, our therapeutic studies suggest that Nrg-1β1 treatment offers an extended therapeutic time window in EAE and can exert beneficial effects under different treatment paradigms. However, continuous administration of Nrg-1β1 appears to be critical for its beneficial effects in improving functional recovery in EAE.

Nrg-1β1 ameliorates EAE by limiting leucocyte infiltration and inflammation foci

To unravel the potential mechanisms by which Nrg-1β1 treatment improves the neurological outcomes of EAE, we performed an array of histopathological, cellular and molecular analyses. Our overall histopathological analysis of haematoxylin and eosin-Luxol fast blue stained spinal cord tissue after 2 weeks of treatment showed that Nrg-1β1 treatment significantly reduced the number of lesions (44%) and lesion area (60%) in the EAE mice as compared to the vehicle group (Fig. 3A–C). EAE lesions were identified by increased cellularity indicative of inflammatory infiltration. To confirm whether reduced EAE lesion size reflected a decrease in leucocyte infiltration, we used immunostaining for CD45 (a general leucocyte marker) and laminin (marking vasculature and perivascular regions) in EAE lesions

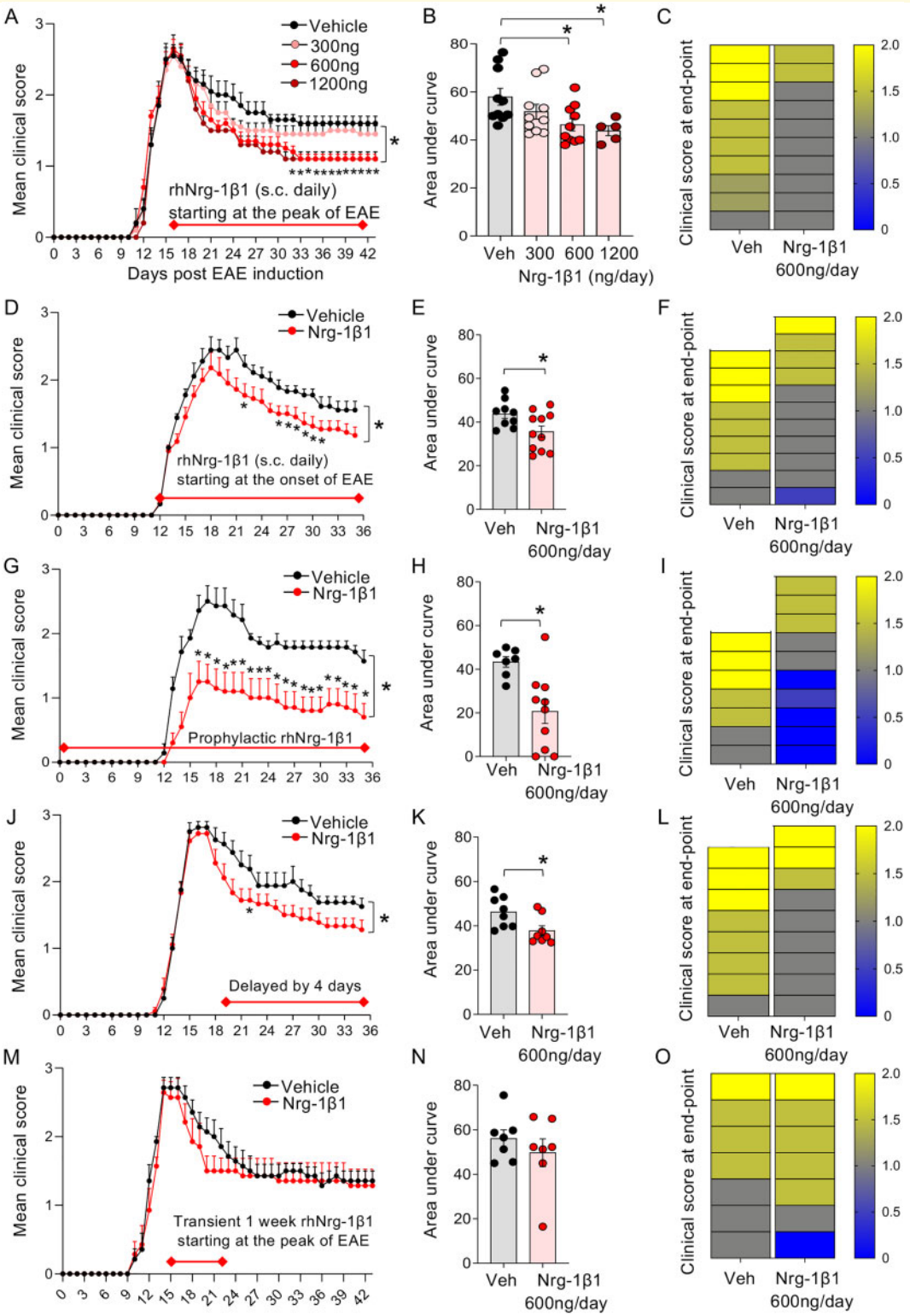

Figure 2 Nrg-1β1 treatment ameliorates neurological disability in EAE mice. (**A**) Mice were assessed daily for EAE symptoms on the basis of tail and hind limb functional deficits. Treatment with recombinant human Nrg-1β1 peptide (300 ng/day, 600 ng/day and 1200 ng/day) was administered subcutaneously (s.c.) starting at the peak of the disease (Day 16 post induction) for 4 weeks. Nrg-1β1 treatment improved functional deficits in a dose dependent manner in EAE mice. Daily clinical scores were expressed as mean ± SEM, $^*P < 0.05$. Two-way-ANOVA followed by Holm-Sidak *post hoc* test. (**B**) Cumulative disease burden for each animal was calculated as area under the curve. The 600 ng/day and 1200 ng/day Nrg-1β1-treated groups showed significant reduction in their mean cumulative disease burden as compared to the vehicle-treated group. $^*P < 0.05$. $n = 10$ for vehicle, 300 ng/day and 600 ng/day Nrg-1β1 groups. $n = 5$ for 1200 ng/day Nrg-1β1 group. (**C**) Clinical score of each mouse in vehicle and NRG1 treatment group (600 ng/day) are plotted as a heat map. Sustained daily Nrg-1β1 treatment (600 ng/day) significantly

(continued)

(Fig. 3D and E). We observed a considerable reduction in density of $CD45^+$ cells in perivascular cuff and EAE lesions in the spinal cord. Our quantification of $CD45^+/DAPI^+$ cells within EAE lesions showed a significant reduction (38%) in infiltrating leucocytes in the Nrg-1β1 treated group in relation to vehicle treated animals (Fig. 3F). To unravel the underlying mechanisms by which Nrg-1β1 inhibits leucocyte infiltration into the spinal cord, we studied the expression pattern of known chemokines involved in this process by using electro-chemiluminescence-based multiplex ELISA at 2, 7 and 14 dpp. These timelines represent early and delayed leucocyte response in EAE progression. We found that Nrg-1β1 treatment modulates key chemokines involved in recruitment of neutrophils (CXCL1/2, keratinocyte chemoattractant KC/human growth-regulated oncogene KC-GRO), monocytes (MCP1, monocyte chemotactic protein 1) and T cells (CXCL10, interferon-γ-inducing protein 10) (Fig. 3 G–I). For CXCL1/2, we detected a significant reduction (42% and 52%) with Nrg-1β1 at 7 and 14 dpp, respectively, compared with vehicle treated EAE animals (Fig. 3G), while MCP1 was significantly reduced (54%) at earlier time point (2 dpp) and showed only modest effects at 7 and 14 dpp (Fig. 3H). Interestingly, Nrg-1β1 treatment significantly reduced CXCL10 (>65%) at all the examined time points (Fig. 3I). These findings indicate that limiting leucocyte infiltration into the CNS tissue is one immunomodulatory mechanism by which Nrg-1β1 attenuates EAE severity.

In EAE, matrix metalloproteinases (MMPs), in particular MMP9, disrupt the integrity of blood–CNS barrier and thereby facilitate leucocyte infiltration into the CNS tissue (Yong *et al.*, 2001). Thus, we asked whether Nrg-1β1 treatment influences MMP activity in EAE. Through gelatin zymography, we assessed the enzymatic activity of MMP2 and MMP9 within the spinal cord tissue. We demonstrate that Nrg-1β1 treatment significantly attenuated the EAE-induced increase in MMP9 activity by 42% (Fig. 3J and L). However, we found no apparent changes in MMP2 activity as the result of EAE (Fig. 3K and L). Recent studies have implicated CSPGs in pathogenesis of EAE and multiple sclerosis (Stephenson *et al.*, 2018, 2019; Stephenson and Yong, 2018). Upregulation of CSPGs after EAE is shown to promote accumulation of leucocytes in the perivascular cuff and facilitate their infiltration into the EAE lesions (Stephenson *et al.*, 2018). Our immunohistochemical analysis of EAE lesions confirmed co-localization of CSPGs with microglia and macrophages (Iba-1+) as well as astrocytes (GFAP+), as expected (Fig. 4A and B). However, activated microglia and macrophages seemed to show a greater degree of co-localization with CSPGs in EAE lesions than astrocytes. Our quantitative immunofluorescence intensity analysis of CSPGs showed that Nrg-1β1 treatment reduced the EAE induced upregulation of CSPGs in the spinal cord by 40% (Fig. 4A and C). These results were corroborated with our complementary slot blot analysis of CSPGs (Fig. 4D). Our findings indicate that availability of Nrg-1β1 attenuates leucocyte infiltration in EAE through several mechanisms including modulation of chemokines, MMP9 and CSPGs.

Availability of Nrg-1β1 positively regulates innate immune response in EAE

CNS microglia and monocyte-derived macrophages are components of the innate immune response that play a pivotal role in EAE pathogenesis (Ajami *et al.*, 2011; Jiang *et al.*, 2014; Moline-Velazquez *et al.*, 2016). We sought to determine whether Nrg-1β1 treatment influences the response of microglia and monocyte-derived macrophages. Our flow cytometry of spinal cord tissue at 2 dpp (Supplementary Fig. 3) and 7 dpp (Fig. 5) showed no significant change in the overall presence of $CD3^-/CD11b^+$ population between Nrg-1β1 and vehicle treated EAE mice suggesting the number of microglia and macrophage remains relatively unchanged under Nrg-1β1 therapy (Fig. 5A; the gating strategy is shown in Supplementary Figs 4 and 5). This was confirmed by complementary immunohistochemical analysis of $Iba\text{-}1^+$ microglia and macrophages in the spinal cord of EAE mice, which also remained unchanged (Fig. 5B). To examine the effect of Nrg-1β1 treatment on resident microglial, we performed cell counts for the microglia specific marker, TMEM119 and found no significant difference in the number of microglia in EAE lesions of Nrg-1β1 versus vehicle treated mice (Fig. 5C and D). Next, we specifically examined circulating monocytes and monocyte derived macrophages by flow cytometry in the blood ($CD3^-/CD11c^{lo}/CD11b^{hi}/Ly6g^-/NK1.1^-$) and spinal cord tissue ($CD3^-/CD49e^+/CD11c^-/Ly6c^+$), respectively. Intriguingly, we detected a significant reduction in both circulating (46%) and infiltrating (28%) monocytes with Nrg-1β1 treatment at 7 dpp (Fig. 5E and F), while they remained unaltered at 2 dpp (Supplementary Fig. 3B). These findings suggest that the overall reduction in leucocytes that we detected in EAE lesions after 7 days of Nrg-1β1 treatment reflects, at least in part, its suppressive effects on monocyte expansion and/or infiltration into the CNS.

Figure 2 Continued

improved clinical score and reduced the cumulative burden of disease when administered at different paradigms including symptomatically at the onset of EAE (**D**–**F**), prophylactically at the time of EAE induction (**G**–**I**), and delayed at 4 days after reaching the peak of the disease (**J**–**L**). (**M**–**O**) Transient Nrg-1β1 therapy for 7 days starting at peak of EAE did not confer beneficial effects when assessed. Clinical scores are expressed as average (mean ± SEM). *$P < 0.05$. Two-way-ANOVA followed by Holm-Sidak *post hoc* test. $n = 7$–10. *$P < 0.05$; Mann-Whitney non-parametric test for area under curve graph statistics.

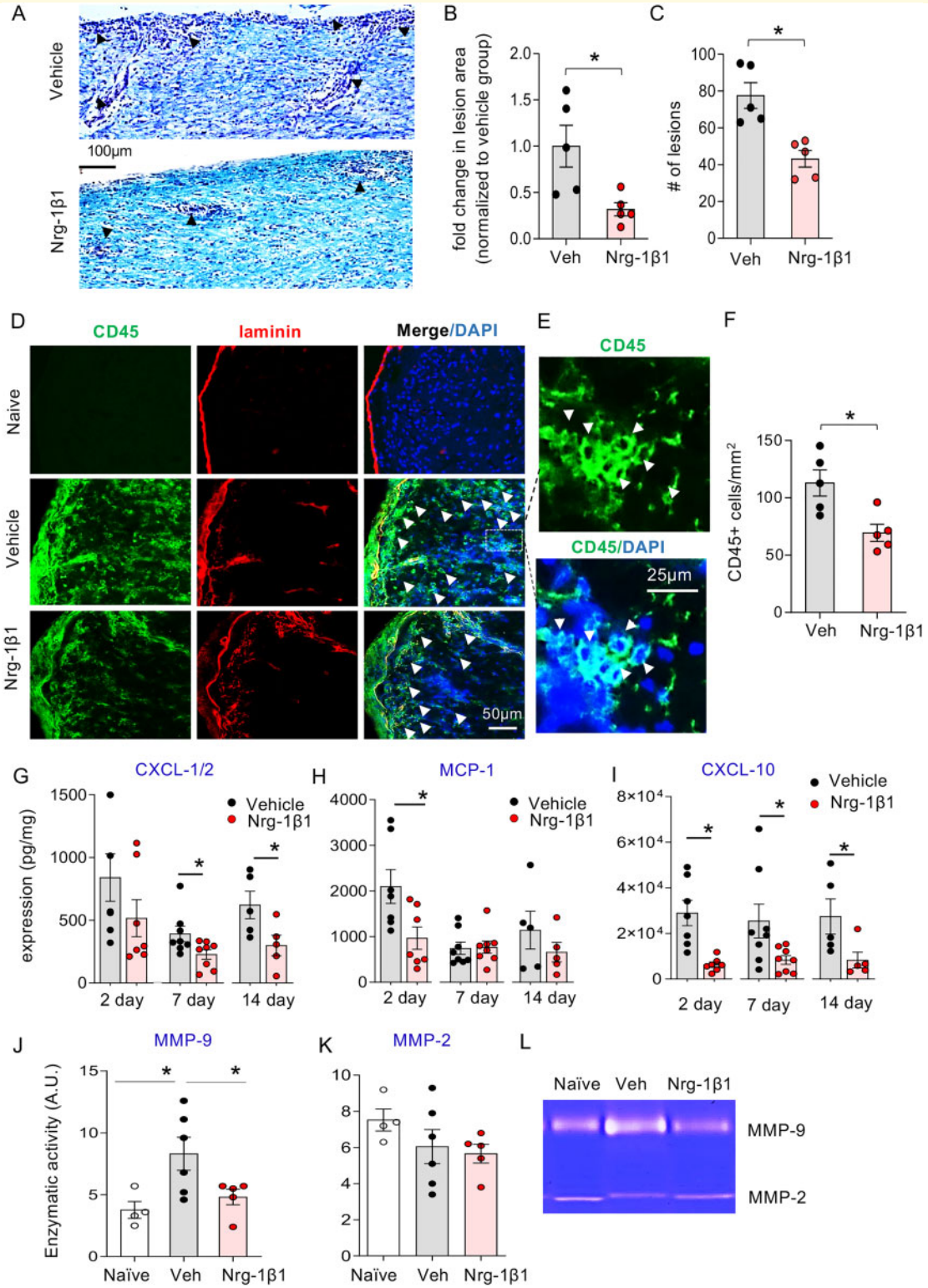

Figure 3 Nrg-1β1 treatment attenuates leucocyte infiltration and inflammation foci in the spinal cord of EAE mice. (**A**) Representative images of haematoxylin and eosin-Luxol fast blue stained spinal cord tissue show active inflammatory and demyelinating lesions (black arrows) in the white matter (WM) from vehicle and Nrg-1β1 treated animals 2 weeks after peak treatment. (**B** and **C**) Nrg-1β1 treatment significantly reduced the area and number of EAE lesions as compared to the vehicle group. *$P < 0.05$; Student's *t*-test. $n = 5$. (**D**) Representative images of perivascular and spinal cord tissue stained with the leucocyte marker CD45 and laminin in naïve, vehicle and Nrg-1β1 treated group. (**E**) Higher magnified images of $CD45^+/DAPI^+$ in the spinal cord. (**F**) Infiltrating $CD45^+$ cells were significantly reduced after Nrg-1β1 treatment as compared to vehicle group. *$P < 0.05$; Student's *t*-test. $n = 5$. (**G**–**I**) Multiplex Mesoscale ELISA at 2, 7 and 14 dpp revealed that Nrg-1β1 treatment significantly reduces the expression levels of chemokines involved in (**G**) recruitment of neutrophils, CXCL1/2 (KC-GRO), (**H**) MCP1 and

(continued)

Since the phenotype of microglia and monocyte-derived macrophages has a significant impact on the neuroinflammatory landscape in EAE, we next studied whether Nrg-1β1 treatment modulates the immune properties of these cells in the spinal cord of EAE mice. Our flow cytometry assessment identified a significant reduction (40%) in $CD3^-/CD11b^+/CD80^+$ pro-inflammatory 'M1' type microglia and macrophages with Nrg-1β1 treatment as compared to vehicle group at 7 dpp. However, there was no significant change at 2 dpp time point analysis (Fig. 5G and Supplementary Fig. 3C; gating strategy in Supplementary Fig. 5). This response was also accompanied by a striking increase in $CD3^-/CD11b^+/CD206^+$ anti-inflammatory 'M2'-like microglia and macrophages under Nrg-1β1 treatment at both 2 dpp (317%) and 7 dpp (274%) (Fig. 5H and Supplementary Fig. 3D). We further assessed whether Nrg-1β1 influences the phenotype of infiltrating monocytes in the EAE spinal cord. Flow cytometry indicated Nrg-1β1 treatment significantly decreased the number of monocyte derived M1-like macrophages ($CD3^-/CD49e^+/CD80^+$) by 34% at 7 dpp, while promoting M2-like macrophages ($CD3^-/CD49e^+/CD206^+$) at both 2 dpp and 7 dpp (28% and 90%, respectively) (Fig. 5I, J, and Supplementary Fig. 3F). However, there was no change in monocyte derived M1-like macrophages with Nrg-1β1 treatment at the 2 dpp time point (Supplementary Fig. 3E; gating strategy in Supplementary Fig. 5). Our complementary immunohistochemistry also verified this M1/M2 phenotype shift in the spinal cord of Nrg-1β1 treated EAE mice (Fig. 5K and L). Since microglia and macrophages also act as antigen presenting cells (APCs) in EAE, we studied the effects of Nrg-1β1 on their phenotype in this context. Interestingly, while Nrg-1β1 treatment significantly reduced (33%) the overall number of $CD3^-/IA/IE^+$ APCs in EAE lesions, it did not alter $CD3^-/CD11b^{+/}IA/IE^+$ microglia/macrophage APCs (Fig. 5M and Supplementary Fig. 6A; gating strategy in Supplementary Fig. 7).

Cytokine release profile of immune cells reflects their functional impact on the neuroinflammatory response in EAE. Thus, we also conducted a time course analysis of some key cytokines associated with pro-inflammatory M1-like cells in the spinal cord of EAE mice using multiplex mesoscale platform. We found that Nrg-1β1 treatment dramatically reduced the release of interleukin (IL)-1β at 2- and 7-day post EAE peak. IL-6 and tumor necrosis factor alpha (TNF-α) levels were also declined significantly in the spinal cord after Nrg-1β1 treatment at all examined time points (2, 7, 14 dpp), as compared to the vehicle group (Fig. 5N–P). However, spinal cord levels of the anti-inflammatory cytokine IL-10 remained unchanged with Nrg-1β1 treatment at all time points (Supplementary Fig. 6B).

Reactive oxygen species derived from macrophages are involved in EAE and multiple sclerosis pathogenesis (Bizzozero *et al.*, 2005; Nikic *et al.*, 2011; Fischer *et al.*, 2013). Thus, we asked whether the decrease in M1 macrophages would be associated with reduced reactive oxygen species levels in the spinal cord tissue. We assessed reactive oxygen species levels in the spinal cord of EAE mice with red fluorescent ethidium signal intensity generated by oxidation of DHE. EAE expectedly induced a robust increase (73%) in the basal levels of reactive oxygen species, which was significantly reduced in Nrg-1β1 treated EAE mice (27%) (Fig. 5Q and R). We also asked whether reduction in M1 macrophages and reactive oxygen species levels may attenuate EAE induced lipid peroxidation during oxidative stress. Slot blot analysis of oxidized lipids marker, E06, showed high levels of lipid peroxidation in the EAE mice at 14 dpp, as compared to its non-detectable levels in non-EAE naive animals. Nrg-1β1 treatment remarkably attenuated oxidized lipids (80%) as compared to vehicle treated EAE mice (Fig. 5S). Collectively, our findings indicate that Nrg-1β1 regulates monocyte expansion and infiltration peripherally in EAE and fosters a phenotype in macrophages and microglia in the CNS that supports resolution of the pro-inflammatory landscape in EAE mice.

Nrg-1β1 mitigates T helper 1 response directly and indirectly by modulating macrophages

T-cell-triggered autoimmunity is a major mechanism of EAE and multiple sclerosis pathogenesis (Zamvil and Steinman, 1990). Therefore, we next investigated whether Nrg-1β1 modulates EAE pathogenesis and resolution by influencing T helper cell population. Flow cytometry at 2 and 7 dpp identified no change in total number of $CD3^+/CD4^+$ T cells in the blood or spinal cord of EAE mice suggesting Nrg-1β1 did not affect overall T cell expansion peripherally, nor their presence in the spinal cord (Fig. 6A, B and Supplementary Fig. 3G). However, Nrg-1β1 treatment appeared to influence T cell phenotype in EAE lesion as we detected a significant reduction (18.73%) in the Th1 interferon gamma cytotoxic cells ($CD4^+/IFN\gamma^+$) population in the spinal cord of EAE

Figure 3 Continued

(**I**) chemokine for T cells, CXCL10. Data represent mean ± SEM, $^*P < 0.05$; Student's *t*-test. $n = 4–8$. (**J**) Gelatin zymography for MMP2 and MMP9 activity was performed on the spinal cord lysate samples of EAE mice treated with vehicle or Nrg-1β1. MMP9 activity was significantly elevated as the result of EAE, which was significantly reduced by Nrg-1β1 treatment to a level close to the basal level detected in naïve non-EAE spinal cord. (**K**) No alteration in activity of MMP2 was observed in vehicle or Nrg-1β1 treated group as compared to the naïve group. (**L**) Representative gelatin zymogram of MMP2 and MMP9 activity in the spinal cord lysate samples of EAE mice treated with vehicle or Nrg-1β1. Data represent mean fold change in expression ± SEM normalized to the naïve group. $^*P < 0.05$. One-way ANOVA followed by Holm-Sidak *post hoc* test. $n = 4–6$.

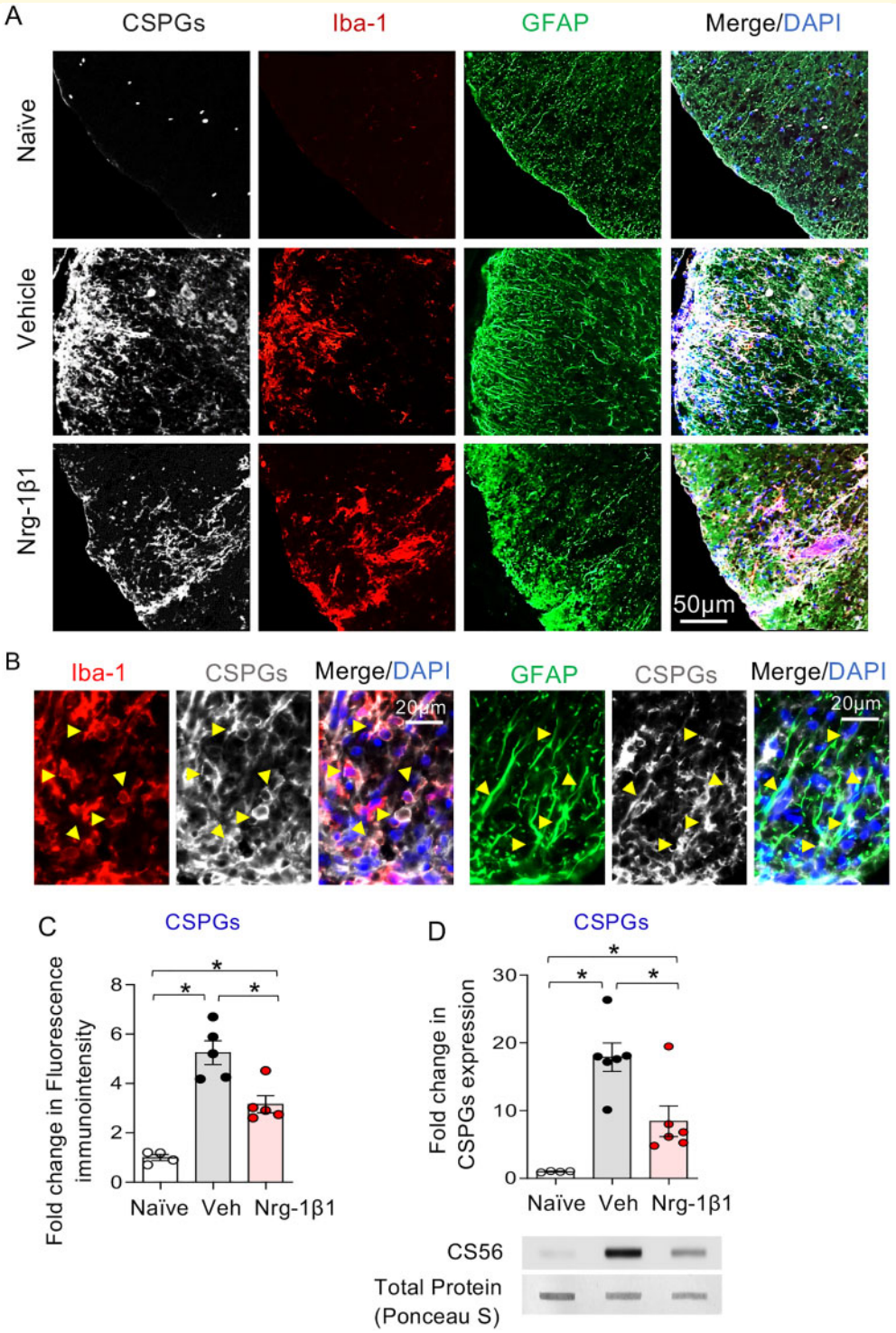

Figure 4 Nrg-1β1 treatment attenuates the expression of CSPGs in EAE lesions. (**A**) Representative images of immunohistochemical staining of CSPGs, microglia/macrophage (Iba-1) and astrocytes (GFAP) in naïve, vehicle and Nrg-1β1 treated groups. Treatments were administered for 2 weeks starting at peak of the disease. (**B**) CSPGs were highly upregulated in association with both astrocytes and microglia/macrophages as demonstrated by co-labelling with Iba-1 and GFAP in the high-magnification images of marked area. Yellow arrows show co-labelling with Iba-1 and GFAP, respectively. (**C** and **D**) Quantitative immunofluorescence intensity and slot blot analysis showed Nrg-1β1 treatment significantly abated the EAE-induced expression of CSPGs. Data represent mean fold change in expression ± SEM normalized to the naïve group. *$P < 0.05$. One-way ANOVA followed by Holm-Sidak *post hoc* test. $n = 4–5$.

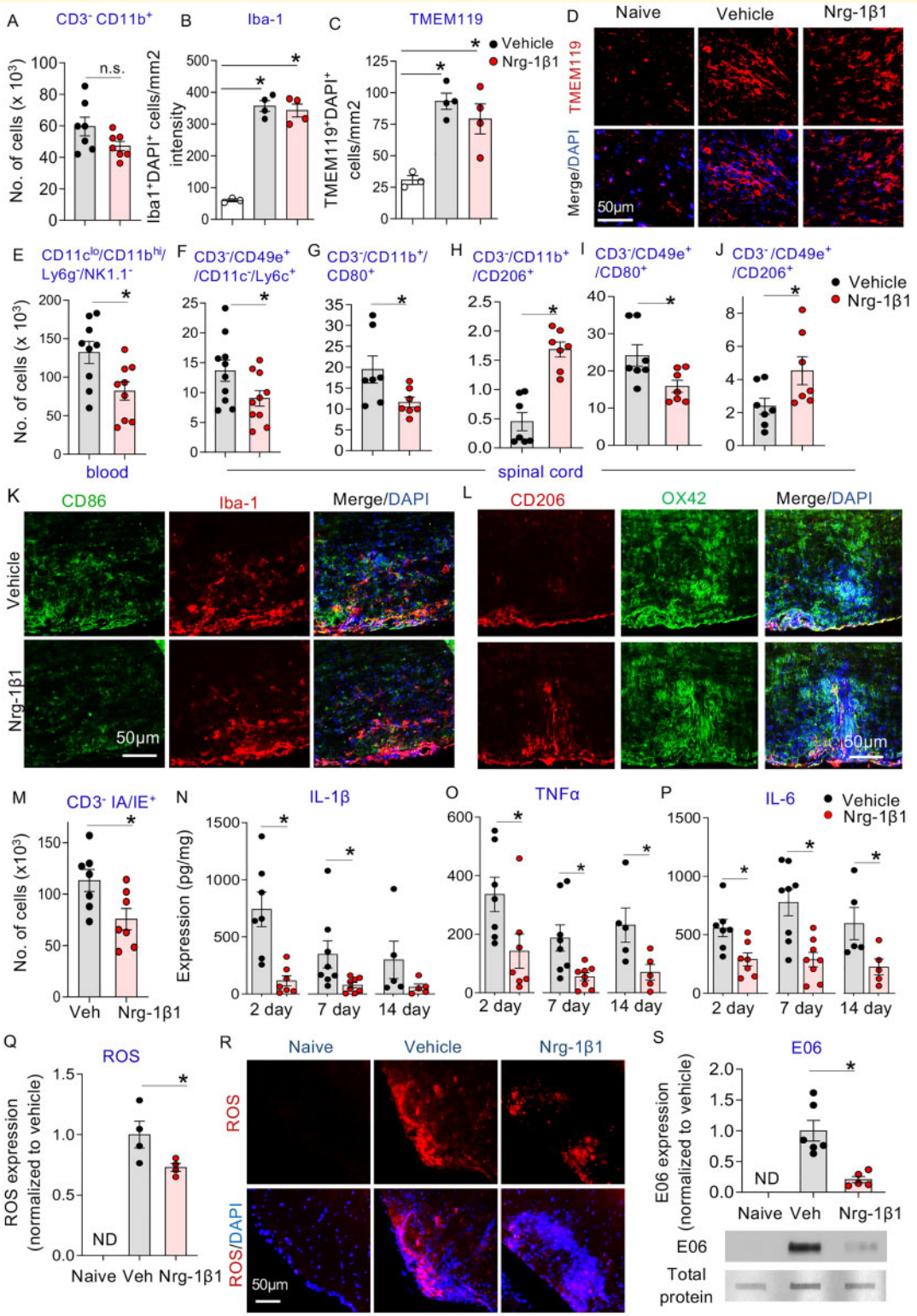

Figure 5 Nrg-1β1 suppresses monocyte expansion and infiltration and attenuates pro-inflammatory phenotype of microglia and macrophages in EAE mice. (**A**) Flow cytometric analysis of spinal cord from vehicle and Nrg-1β1 treated animals at 7 dpp showed that Nrg-1β1 did not change the overall population of $CD3^{-}/CD11b^{+}$ microglia and macrophages. (**B** and **C**) Immunohistochemical cell density analysis of microglia/macrophage common marker, Iba-1 and microglia specific marker TMEM119 also confirmed that Nrg-1β1 did not alter the recruitment/activation of macrophages and resident microglia in the spinal cord as compared to the vehicle EAE groups. $^{*}P < 0.05$. One-way ANOVA, $n = 3$–4. (**D**) Representative images of TMEM119 immunostaining from spinal cord lesions of naïve, vehicle and NRG1 treated groups are shown. (**E** and **F**) Nrg-1β1 treatment significantly reduced circulating monocytes ($CD11c^{lo}/CD11b^{hi}/Ly6g^{-}/NK1.1^{-}$) in the blood and infiltrating macrophages ($CD3^{-}/CD49e^{+}/CD11c^{-}/Ly6c^{+}$) in the spinal cord of Nrg-1β1 treated animals as compared to vehicle group. (**G** and **H**)

(continued)

mice at the 7 dpp time point (Fig. 6C, D and Supplementary Fig. 3H; gating strategy in Supplementary Fig. 8). Of note, the number of circulating Th1 $CD4^+/IFN\gamma^+$ cell population in the blood remained unaffected with Nrg-1β1 treatment, suggesting that the effects of Nrg-1β1 on T cell phenotype were more specific to the environment of EAE spinal cord (Fig. 6C). Nrg-1β1 effects were also accompanied by a significant reduction in the spinal cord levels of $IFN\gamma^+$, IL-2 and IL-16 under NRG1 treatment at 2 dpp and/or 7 dpp (Fig. 6E–G). These cytokines are key mediators of Th1 cell differentiation and function in EAE and multiple sclerosis (Skundric *et al.*, 2015). We also studied the Th17 response, another key driver of EAE pathogenesis (Rostami and Ciric, 2013). However, flow cytometry of the effector Th17 population ($CD4^+/IL\text{-}17^+$) showed that Nrg-1β1 treatment did not alter effector Th17 population in the blood or the spinal cord of EAE mice at 2 dpp and 7 dpp (Fig. 6H, I and Supplementary Fig. 3I). In contrast, anti-inflammatory regulatory T cells ($CD4^+/CD25^+/FR4^+$ and $CD4^+/CD25^+/FoxP3^+$) were significantly elevated at 2 and 7 dpp as the result of Nrg-1β1 treatment (Fig. 6J, K and Supplementary Fig. 3J and K). These EAE findings suggest that although availability of Nrg-1β1 does not suppress the overall recruitment of T cells peripherally or in the EAE lesions, it fosters a more balanced T-cell response by suppressing effector Th1 response while promoting regulatory T-cell populations.

Response and phenotype of T cells in EAE pathogenesis and recovery is highly influenced by their cross-talk with CNS innate immune cells (microglia, macrophages and astrocytes) (Zamvil and Steinman, 1990; Ajami *et al.*, 2011; Olah *et al.*, 2012; Miron *et al.*, 2013; Rostami and Ciric, 2013; Jiang *et al.*, 2014; Brambilla, 2019). Since we found that Nrg-1β1 regulated T-cell phenotype in the spinal cord but not in the blood, we asked whether it influenced T-cell phenotype indirectly through its modulatory effects on astrocytes, microglia and/or monocyte derived macrophages in EAE lesions. Notably, our immunocytochemical assessments confirmed that microglia, macrophages and astrocytes express Nrg-1β1 ligand binding receptors, ERBB2 and ERBB4 (Supplementary Fig. 9). To address this hypothesis, we performed *in vitro* studies. We polarized naïve $CD4^+$ T cells under Th1 and Th17 polarization conditions and subject them to conditioned media from microglia, BMDMs or astrocytes under M0 (control) or M1 ($IFN\gamma$ + LPS treated) conditions with or without Nrg-1β1 treatment. First, we demonstrate that direct treatment with Nrg-1β1 resulted in a reduction in the number of Th1 polarized cells ($CD4^+/IFN\gamma^+$) in a concentration dependent manner, as compared to the control condition (Fig. 6L). Then, we found that conditioned media of M1 BMDM significantly increased Th1 population (45%), while M1 BMDM treated with Nrg-1β1 (200 ng/ml) decreased Th1 cells (26%). Treatment with Nrg-1β1 did not affect the effects of M0 non-activated BMDM cells on Th1 polarization. This demonstrates that availability of Nrg-1β1 can inhibit Th1 polarization by regulating the response of pro-inflammatory M1 macrophages (Fig. 6M). Interestingly, conditioned media of activated M1 microglia or astrocytes conditioned media did not result in any significant change in the population of Th1 polarized cells, suggesting a specific role for macrophages in promoting Th1 response (Fig. 6N and O). Our flow cytometry studies of Th17 polarized cells showed no changes in the number of Th17 effector cells ($CD4^+/IL\text{-}17^+$) neither under Nrg-1β1 nor BMDM or microglia conditioned media (Supplementary Fig. 10A–C), while astrocyte conditioned media itself (both M0 and M1) significantly attenuated the generation of $CD4^+/IL\text{-}17^+$ Th17 cells, although it was irrespective of Nrg-1β1 treatment (Supplementary Fig. 10D). Interestingly, our *in vitro* flow cytometry revealed that Nrg-1β1 (100 ng/ml and 200 ng/ml) also directly reduced (27–35%) Th1 effector cells ($CD4^+/IFN\gamma^+$) under Th17 polarization, confirming our finding in the EAE mice (Supplementary Fig. 10E). Although conditioned media of BMDMs (both M0 and M1) did not affect the number of $CD4^+/IFN\gamma^+$ cells, conditioned media from M0 and M1 microglia significantly attenuated these pro-inflammatory cells. However, this effect was irrespective of Nrg-1β1 treatment (Supplementary Fig. 10F and G). In contrast, conditioned media from activated astrocytes treated with Nrg-1β1 (200 ng/ml) significantly attenuated $CD4^+/IFN\gamma^+$ effector Th1 under Th17 polarization in comparison to conditioned media of non-activated astrocytes treated condition (Supplementary Fig. 10H). Taken together, these findings suggest that Nrg-1β1 primarily regulate the Th1 mediated

Figure 5 Continued

Nrg-1β1 treatment also significantly reduced pro-inflammatory M1 ($CD3^-/CD11b^+/CD80^+$) microglia/macrophages, while promoted an anti-inflammatory M2 ($CD3^-/CD11b^+/CD206^+$) phenotype. (**I** and **J**) Monocyte derived M1 macrophages ($CD3^-/CD49e^+/CD80^+$) were also decreased in the spinal cord Nrg-1β1 treated mice, while 'M2' macrophages were increased ($CD3^-/CD49e^+/CD206^+$). (**K** and **L**) Representative images of EAE spinal cord immunostained with M1 marker (CD80) or 'M2' marker (CD206) co-labelled with microglia/macrophage markers Iba-1 or OX-42, respectively, show the M1 to M2 phenotype shift in microglia/macrophage population in Nrg-1β1 treated group as compared to vehicle group at the 7 dpp time point. (**M**) Flow cytometric assessment showed a significant reduction in total antigen presenting cells ($CD3^-IA/IE^+$) in the EAE spinal cord under Nrg-1β1 treatment as compared to vehicle group. (**N**–**P**) Cytokine analysis by ELISA in spinal cord tissues also revealed Nrg-1β1 treatment significantly reduced pro-inflammatory cytokines IL-1β, TNF-α, and IL-6. (**Q** and **R**) Reactive oxygen species (ROS) was detected in EAE mice by conversion of DHE to ethidium in the spinal cord tissue. EAE resulted in substantial increase in reactive oxygen species levels in the white matter of the spinal cord in which was significantly reduced by Nrg-1β1 treatment. (**S**) Slot blot analysis of oxidized lipids (E06) was performed on spinal cord lysates at 14 days after Nrg-1β1 treatment. EAE induced E06 levels, which was reduced significantly after Nrg-1β1 treatment as compared to the vehicle treated group. Data represent mean ± SEM. $^*P < 0.05$; Student's *t*-test. $n = 6$–8.

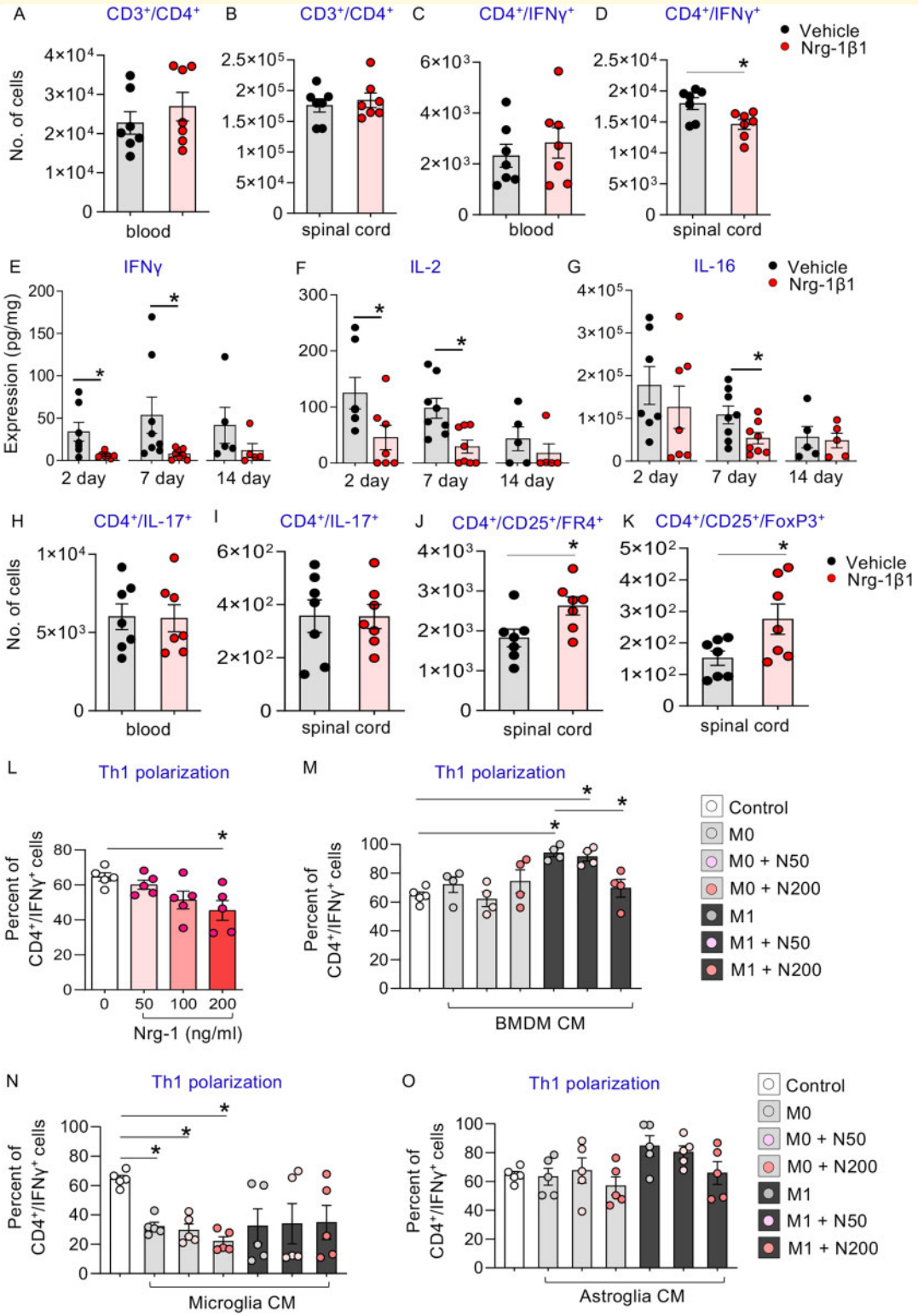

Figure 6 Nrg-1β1 treatment promotes a T regulatory response directly and indirectly by modulating macrophages. (**A** and **B**) Flow-cytometry of vehicle and Nrg-1β1 treated mice after 7 days of treatment revealed no change in the total number of $CD4^+$ T cells in the blood and spinal cord. (**C** and **D**) While the total number of effector T cells ($CD4^+IFN\gamma^+$) remained unchanged in the blood, there was a significant reduction in $CD4^+IFN\gamma^+$ cells in the spinal cord of Nrg-1β1 treated animals as compared to vehicle group. (**E**–**G**) Cytokine assessment in spinal cord tissue with ELISA showed that Nrg-1β1 treatment significantly reduced key drivers of Th1 cell differentiation IFNγ, IL-2 and IL-16. $^*P < 0.05$; Student's *t*-test. $n = 6$–8. (**H** and **I**) Flow-cytometry for $CD4^+IL\text{-}17^+$ effector T cell population after 7 days of Nrg-1β1 treatment did not show any change in this population in the blood or spinal cord. (**J** and **K**) A significant increase in anti-inflammatory T regulatory ($CD4^+/CD25^+/FR4^+$) and $CD4^+/CD25^+/FoxP3^+$ cells was observed with Nrg-1β1 treatment as compared to vehicle group. $^*P < 0.05$;

(continued)

inflammatory response in EAE directly, which appears to be directly and indirectly through modulation of monocytes/macrophages and to some extent astrocytes.

Protemics asserts the impact of Nrg-1β1 on modulating pathways involved in immune response and lipid oxidation in EAE

To validate immune modulatory role of Nrg-1β1 treatment in the EAE, we performed LC-MS/MS based proteomics on the spinal cord tissue of EAE mice at 7 days post treatment, the time point that Nrg-1β1 showed most of its significant regulatory effects. Comparing Nrg-1β1 and vehicle treated groups, we found 342 differentially expressed proteins as the result of Nrg-1β1 therapy (Fig. 7A). Pathway analysis of upregulated and downregulated proteins in Nrg-1β1 treated group with respect to vehicle group unveiled some of the key biological functions related to immune response, lipid oxidation, stress response, apoptotic signalling and mitochondrial/vesicle transport (Fig. 7B). A detailed database search through DAVID software corroborated these findings as immune response, lipid oxidation, cell adhesion and cell differentiation were some of the GO pathways, which significantly enriched in pathway analysis (Fig. 7C). To elaborate on these bioinformatics data, we performed specific pathway analysis using ClueGo software. Reactome and GO database pathway analysis affirmed our ELISA and flow cytometry assessment suggesting the involvement of IL-1, IL-17 and TLR cascades in Nrg-1β1 mediated effects in EAE spinal cord tissue (Supplementary Table 6). Of note, Nrg-1β1 ameliorated the proteins/transcription factors associated with leucocyte trans-endothelial migration, chemokine mediated migration, leucocyte infiltration and macrophage-restricted adhesion molecules, confirmed our cellular assessment that showed availability of Nrg-1β1 inhibits leucocyte infiltration into the CNS during EAE pathogenesis. Interestingly, pathway analysis also affirmed the effects of Nrg-1β1 in attenuating lipid oxidation and fatty acid catabolic processes. Collectively, our immunohistochemical, flow cytometry, cytokine profiling and proteomics data indicate that Nrg-1β1 reduces monocyte extravasation from the periphery thereby abating the Th1 (IFNγ) mediated inflammatory response in the CNS of EAE mice, which results in reduced/delayed EAE symptoms and facilitates the recovery process.

Nrg-1β1 is depleted in active demyelinating plaques of patients with multiple sclerosis

To validate the relevance of our EAE studies to multiple sclerosis pathophysiology, we investigated Nrg-1β1 protein expression in active demyelinating plaques of six multiple sclerosis brain samples. Using haematoxylin and eosin-Luxol fast blue, we first identified multiple sclerosis demyelinating plaques in the white matter (Fig. 8A). We further confirmed demyelinating lesions by reduced immunofluorescence intensity of myelin basic protein (MBP). Our immunohistochemical analysis showed a significant reduction in Nrg-1β1 expression levels within multiple sclerosis plaques as compared to the surrounding normal-appearing white matter (Fig. 8B, C and Supplementary Fig. 11A). To quantify and account for the variability among multiple sclerosis tissue samples, we normalized immunofluorescence intensity values for each lesion to normal-appearing white matter adjacent area of the same sample. Collective analysis of all samples showed that Nrg-1β1 intensity levels was reduced within the multiple sclerosis plaques by 39% as compared to normal-appearing white matter. These results provide evidence supporting a positive association between Nrg-1β1 downregulation and multiple sclerosis pathology (Fig. 8C and Supplementary Table 5).

Nrg-1β1 levels are lower in early multiple sclerosis and are associated with disease progression

We next determined whether downregulated levels of Nrg-1β1 is also detected peripherally in the plasma of multiple sclerosis individuals, as detected in the EAE mice. We analysed Nrg-1β1 levels in plasma samples of multiple sclerosis and normal individuals (Supplementary Table 7). ELISA analysis included normal participants ($n = 30$) and multiple sclerosis individuals of a patient cohort ($n = 136$) presenting three major sub-types of multiple sclerosis including CIS ($n = 11$), RRMS ($n = 113$) and SPMS ($n = 12$). Primary progressive multiple sclerosis (PPMS) type was not included in our analysis because of insufficient samples within the cohort. Our initial analysis comparing plasma level of Nrg-1β1 among normal individuals and all multiple sclerosis patients, regardless of their disease subtype, showed no significant difference (Fig. 8D and Supplementary Table 7). To ascertain

Figure 6 Continued

Student's *t*-test. $n = 6–8$. (**L**–**O**) Naïve $CD4^+$ T cells were polarized *in vitro* under Th1 conditions and were cultured with different concentration of Nrg-1β1, conditioned medium (CM) from normal (M0) and M1 polarized (IFNγ + LPS treated) microglia and BMDMs treated with Nrg-1β1 50 ng/ml or 200 ng/ml for 72 h. (**L**) Flow cytometric assessment revealed higher dose of Nrg-1β1 (200 ng/ml) significantly reduced $CD4^+IFN\gamma^+$ T cells. (**M**) While there was a significant increase in $CD4^+IFN\gamma^+$ cell population under BMDM M1 conditioned media, Nrg-1β1 200 ng/ml treated M1 BMDM conditioned media was able to diminish this increase significantly. (**N**) M0 Microglial conditioned media reduced the total number of Th1 polarized $CD4^+IFN\gamma^+$ T cells, (**O**) while astrocytes conditioned media did not alter Th1 cell population. $^*P < 0.05$. One-way ANOVA followed by Holm-Sidak *post hoc* test. $n = 4–5$.

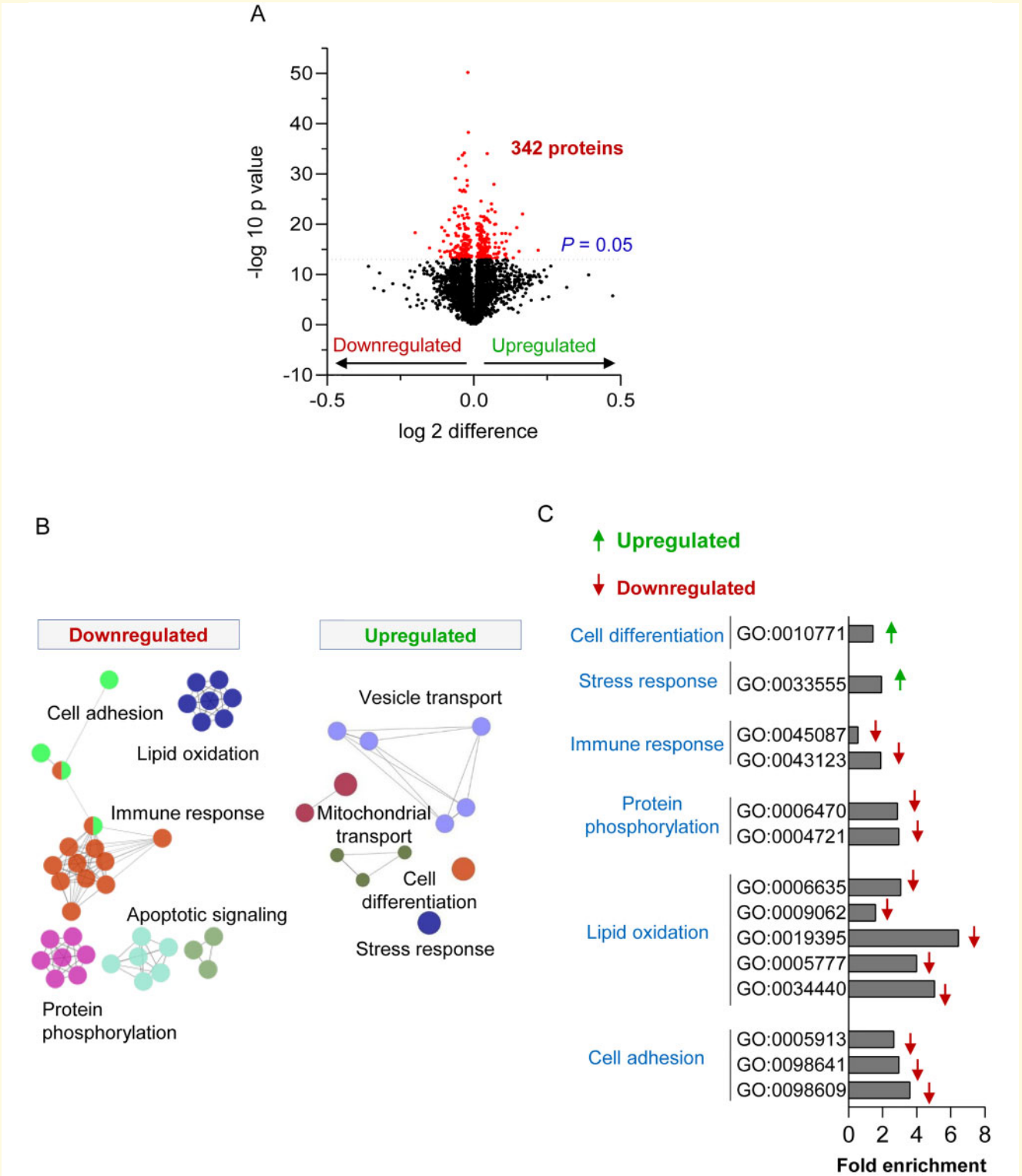

Figure 7 Proteomic analysis asserts that lipid oxidation and immune modulation are key Nrg-1β1 mediated mechanisms in EAE recovery. (**A**) Volcano plot illustrates differentially abundant proteins in the spinal cord of vehicle and Nrg-1β1 treated mice at 7 days post-peak of EAE. The –log10 is plotted against the log2 (fold change). The horizontal line denotes $P = 0.05$, which was set as significance threshold (prior to logarithmic transformation). (**B**) Functional analysis of differentially expressed proteins using ClueGo plug-in in cytoscape software shows the interactions among the significantly different biological functions associated with upregulated and downregulated proteins in this study. Based on the κ score level, biological functions are depicted as coloured nodes linked to related groups. (**C**) Differentially expressed proteins were further analysed using DAVID software. The *x*-axis represents the fold enrichment of each biological function GO term. Only statistically significant GO terms are shown. Key for the GO terms are: GO:0098609, cell, cell adhesion; GO:0098641, cadherin binding involved in cell, cell adhesion; GO:0005913, cell, cell adherens junction; GO:0034440, lipid oxidation; GO:0005777, peroxisome; GO:0019395, fatty acid oxidation;

(continued)

whether Nrg-1β1 expression may vary under disease modifying treatments (DMTs) at the time of sample collection, we also compared Nrg-1β1 levels between DMTs users and non-users. Our analysis indicated no overall difference in Nrg-1β1 levels among all multiple sclerosis patients receiving DMTs as compared to patients who did not receive any treatment (Fig. 8E and Supplementary Table 7). The apparent lack of statistically significant difference in plasma levels of Nrg-1β1 in DMT users and non-user patients could be attributed to the smaller sample size and high variability within the group.

We next determined the plasma levels of Nrg-1β1 among different subtypes of multiple sclerosis. We first plotted Nrg-1β1 levels of multiple sclerosis patients grouped into respective clinical diagnosis for the disease and then subgrouped based on whether they were receiving any DMTs at the time of plasma collection. Intriguingly, we found significantly lower (>50%) plasma levels of Nrg-1β1 in CIS individuals, irrespective of receiving DMTs, in comparison to normal individuals (Fig. 8F, Supplementary Fig. 11B and Supplementary Table 7). Importantly, 55% (6 of 11) of the CIS individuals in this study developed RRMS during a median follow-up of 4 years of the onset of CIS (Supplementary Fig. 11C). Of note, the CIS patients who developed RRMS also showed lower levels of Nrg-1β1 (61% reduction) in plasma, as compared to those who did not develop multiple sclerosis (46% reduction) until their last clinical visit (Fig. 8G). These initial findings provide first evidence suggesting that Nrg-1β1 is dysregulated in early phase of multiple sclerosis.

Next, we investigated the relationship between Nrg-1β1 plasma levels and DMTs among multiple sclerosis subtypes. Interestingly, Nrg-1β1 levels in CIS and SPMS individuals who did not receive DMTs were significantly reduced as compared to normal individuals. Nrg-1β1 levels of RRMS patients, both DMT and non-DMT receiving, were closer to normal individuals than CIS and SPMS individuals (Fig. 8H and Supplementary Table 7). Furthermore, we examined whether Nrg-1β1 plasma levels correlate with the Expanded Disability Status Scale (EDSS) or number of years spent after multiple sclerosis diagnosis and DMTs for each individual (Fig. 8I and Supplementary Fig. 11D). We did not find any statistically significant difference in our analysis due to high degree of variation, although there was an overall reduction in the levels of Nrg-1β1 in individuals who did not receive any DMTs with respect to EDSS score (Fig. 8I). Similarly, there was reduced expression of Nrg-1β1 during early years of multiple sclerosis with respect to EDSS score. However, the difference was not statistically significant as compared to healthy controls (Supplementary Fig. 11D). Overall, the smaller sample size across the groups, higher variability in expression of Nrg-1β1 among multiple sclerosis patients and disparate stage of the disease plausibly led to non-significant outcomes of these analyses. Future studies with larger multiple sclerosis patient sample size and adequate representation of all stages of the disease are warranted to have conclusive evidence about the relation of Nrg-1β1 with disease progression and DMT administration.

Discussion

Currently, little is known about the early endogenous mechanisms that regulate multiple sclerosis autoimmune response at the onset and progression of the disease. Understanding multiple sclerosis mechanisms can aid in identifying disease markers and facilitate clinical assessment, timely diagnosis and treatment of multiple sclerosis. In the present study, utilizing the preclinical EAE mouse model and multiple sclerosis patient samples, we provide new evidence that dysregulation of Nrg-1β1 is associated with multiple sclerosis pathology and is detectable both peripherally in the plasma and centrally in the CNS lesions. Downregulation of Nrg-1β1 precedes EAE symptoms and persists during disease onset and progression, when the immune response is predominantly pro-inflammatory. Importantly, we provide evidence that downregulation of Nrg-1β1 has impact on EAE immunopathogenesis, as therapeutic restoration of Nrg-1β1 in the EAE mice delayed disease onset and attenuated clinical severity of EAE. Relevance of these findings to multiple sclerosis was corroborated by detecting lower levels of Nrg-1β1 in the plasma of CIS individuals at the onset of multiple sclerosis compared to normal individuals. Capitalizing on these new findings, we propose that downregulation of Nrg-1β1 is a disease characteristic in early multiple sclerosis and its reduced levels may indicate disease severity. This notion is furthered supported by our findings that multiple sclerosis patients whose disease was regulated with DMTs had a more normal level of Nrg-1β1 in their plasma. While efforts are being made to identify early disease markers for multiple sclerosis or to develop treatments that can prevent or delay progression to definite multiple sclerosis (Metz *et al.*, 2017), Nrg-1β1 appears to be a promising target for further investigations in this direction.

NRG1 is well-known for its diverse roles in the development and physiology of the nervous system (Kataria *et al.*, 2019). The Nrg-1β1 isoform is predominantly expressed in the nervous system (Nave and Salzer, 2006). In the CNS, NRG1 is highly expressed by neurons and is axonally-

Figure 7 Continued
GO:0009062, fatty acid catabolic process; GO:0006635, fatty acid beta, oxidation; GO:0004721, phosphoprotein phosphatase activity; GO:0006470, protein dephosphorylation; GO:0043123, positive regulation of I, kappaB kinase/NF, kappaB signaling; GO:0045087, innate immune response; GO:0033555, multicellular organismal response to stress; GO:0019771, negative regulation of cell morphogenesis involved in differentiation.

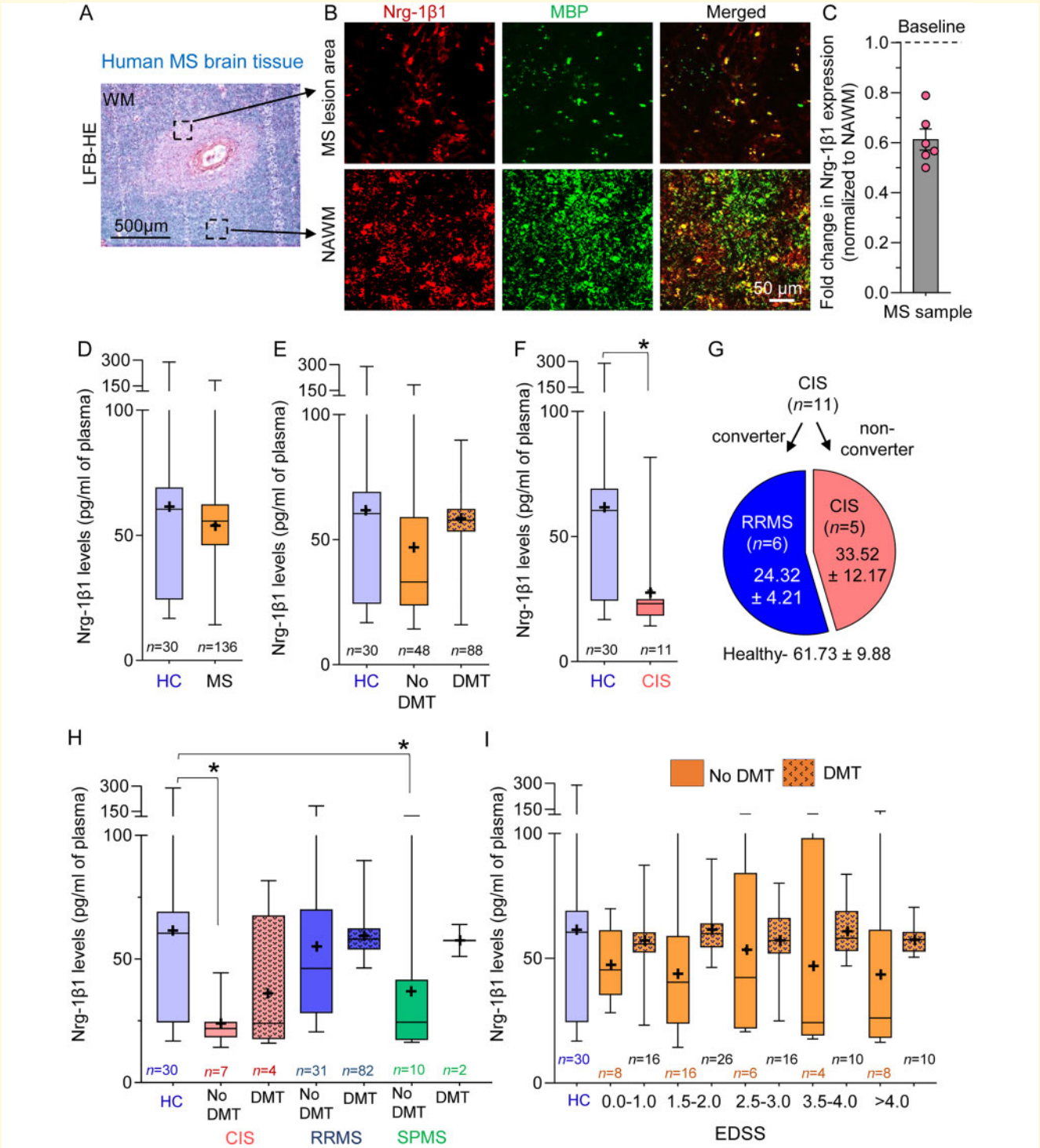

Figure 8 Nrg-1β1 is depleted in plasma and brain lesions of multiple sclerosis patients. (**A**) Post-mortem brain samples from multiple sclerosis patients were stained for Luxol fast blue-haematoxylin and eosin (HE-LFB) to identify demyelinating lesion in the white matter (**B**) Representative images of normal-appearing white matter (NAWM) or lesion area from multiple sclerosis (MS) brain sections stained with antibodies against Nrg-1β1 and MBP. (**C**) Quantification for Nrg-1β1 immunofluorescence intensity was performed from six different multiple sclerosis brain samples comparing the Nrg-1β1 intensity in lesion to the normal-appearing white matter from same section. Values are represented as fold change in intensity normalized to normal-appearing white matter for each sample which is shown as dotted baseline. There was 39% reduction in Nrg-1β1 expression within multiple sclerosis lesions as compared to the adjacent normal-appearing white matter. (**D**–**G**) ELISA was performed for Nrg-1β1 on human plasma samples from normal individuals and multiple sclerosis patients. (**D**) Box plots show range of Nrg-1β1 levels (minimum to maximum) in normal controls (HC, $n = 30$) and multiple sclerosis patients ($n = 136$). (**E**) multiple sclerosis patients were categorized into disease modifying therapy (DMT, $n = 88$) or no DMT ($n = 48$) users at the time of sample collection. Nrg-1β1 levels of DMT users are indicated with dotted box plots. (**F**) CIS individuals showed a significant reduction in Nrg-1β1 levels in plasma as compared to normal

(continued)

localized (Michailov *et al.*, 2004; Gauthier *et al.*, 2013). Likewise, oligodendrocytes express NRG1 in the injured and healthy rodent spinal cord tissue (Gauthier *et al.*, 2013; Bartus *et al.*, 2016). NRG1 is also expressed by astrocytes to a lesser extent, where intracellular cAMP levels and protein kinase C (PKC) signalling pathways have been shown to regulate its expression *in vitro* (Tokita *et al.*, 2001). Expression of *Nrg1* mRNA has been also reported in peripheral blood mononuclear cells (PBMCs) and in mouse brain (Tokita *et al.*, 2001; Ikawa *et al.*, 2017), although our previous studies did not show a detectable level of NRG1 protein in microglia/macrophage in the spinal cord (Gauthier *et al.*, 2013). All known secreted isoforms of NRG1 contain a heparin-binding domain that binds to heparan sulphate proteoglycan and acts as a highly specific targeting mechanism to deliver NRG1 to the extracellular matrix of sites where it is needed, such as developing white matter tracts of the spinal cord and the basal lamina of neuromuscular synapses (Loeb *et al.*, 1998, 1999). Interestingly, NRG1 precursors are produced in cortical neurons, while soluble NRG1 ligand becomes concentrated within the extracellular matrix of white matter, where it can be released into the CSF (Pankonin *et al.*, 2009). Although the underlying cause of Nrg-1β1 downregulation in EAE lesions needs further elucidation, our data suggest its reduction in EAE lesion may reflect degeneration of its primary sources, axons and oligodendrocytes. This observation is reminiscent of our previous studies in traumatic spinal cord injury and lysolecithin (LPC)-induced focal demyelinating lesions that also showed a long lasting reduction in Nrg-1β1 protein expression in white matter lesions of the spinal cord (Gauthier *et al.*, 2013; Kataria *et al.*, 2018). An early study also reported absence of NRG1 in active multiple sclerosis lesions, which was attributed to astrocytes, although the conclusion was made qualitatively (Viehover *et al.*, 2001). Importantly, we have shown for the first time that Nrg-1β1 was more robustly but transiently downregulated peripherally in the spleen and blood of EAE mice during the presymptomatic, onset and peak of the disease, as compared to its persistent but less severe dysregulation in the spinal cord. Further investigations are needed to determine how Nrg-1β1 is transiently depleted in the spleen and blood circulation in early and acute stages of EAE. However, our data support the plausibility of an active suppression of Nrg-1β1 expression rather than extravasation of Nrg-1β1 expressing leucocytes to the CNS, as the decline was simultaneously observed in the EAE lesions. Nonetheless, transient dysregulation of Nrg-1β1 in the spleen and blood during EAE pathogenesis points to its importance as an early disease characteristic and a potential immunotherapeutic target.

Therapeutically, we demonstrate that subcutaneous rhNrg-1β1 treatment delayed EAE onset and alleviated disease progression and severity, when administered prophylactically, symptomatically, acutely or chronically. An early study by Canella and colleagues in 1998 also examined the therapeutic effects of administering glial growth factor 2 (GGF2, a 40 kDa isoform of NRG1), in a chronic relapsing SJL/J mouse model of EAE (Cannella *et al.*, 1998). These studies showed that subcutaneous administration of 2 mg/kg dose of rhGGF2 acutely at the time of EAE induction delayed EAE symptoms and significantly reduced relapses. Treatment at the peak of disease also reduced relapses; however, it had no apparent effects on mean clinical scores in the EAE mice. Of note, effective dose of rhNrg-1β1 for EAE neurological recovery in our study was 600 ng/day/mouse or 30 μg/kg, which is much lower compared to the range of 0.2–2 mg/kg dose of rhGGF2 in these studies (Cannella *et al.*, 1998). Beneficial effects of rhGGF2 on clinical recovery was accompanied by improved remyelination in EAE mice. This work, however, did not study either the expression profile of GGF2 during the course of EAE or its role in pathogenesis of EAE. Thus, it would be intriguing to know whether GGF2 follows the same expression profile and pathological characteristics centrally and peripherally in EAE and multiple sclerosis as what we have identified for Nrg-1β1 in our studies

We have uncovered that the beneficial therapeutic effect of Nrg-1β1 is associated with several immune regulatory mechanisms in EAE. Regulation of monocyte response appeared to be a major mechanism, as Nrg-1β1 treatment specifically suppressed circulating monocytes and reduced their infiltration into EAE lesions, while having had no apparent effects on the number of circulating or infiltrated T cells. These results were well supported by our chemokine profiling in which key monocyte chemoattractants, CXCL1/2, CXCL10 and MCP1, were significantly reduced in Nrg-1β1 treated EAE mice. It is well-established that pro-inflammatory monocyte-derived macrophages accumulate in EAE lesions

Figure 8 Continued

individual samples. *$P < 0.05$; Mann-Whitney U-test. (**G**) Nrg-1β1 plasma levels of CIS individuals were further categorized based on their subsequent diagnosis of multiple sclerosis (RRMS) in the follow-up years and compared to normal patient samples. Six of 11 CIS patients converted to RRMS and represented lower levels of Nrg-1β1 as compared to those who did not progress to multiple sclerosis (non-converter). (**H**) Multiple sclerosis patients were further categorized based on clinical diagnosis into different multiple sclerosis type/stage and whether they received any DMT or not (NDMT). Nrg-1β1 levels in plasma samples from CIS ($n = 11$; NDMT = 7, DMT = 4), RRMS ($n = 113$; NDMT = 31, DMT = 82) and SPMS ($n = 12$; NDMT = 10, DMT = 2) were analysed. Nrg-1β1 levels in CIS and SPMS patients who did not receive DMT were significantly reduced as compared to normal individuals. *$P < 0.05$; Mann-Whitney U-test. (**I**) Nrg-1β1 plasma levels of normal individuals and multiple sclerosis patients (with or without DMT) were analysed against their EDSS. Nrg-1β1 levels were stable in DMT receiving patients irrespective of EDSS score, while multiple sclerosis patients without any DMT demonstrated lower levels of Nrg-1β1 as compared to normal individuals. The plus symbol indicates the mean value of Nrg-1β1 levels among analysed samples.

during onset and peak of the disease (Jiang *et al.*, 2014; Moline-Velazquez *et al.*, 2016) and drive autoimmune mediated demyelination by producing cytokines and presenting myelin epitopes to activating $CD4^+$ T cells (McMahon *et al.*, 2005; van Zwam *et al.*, 2011; Sosa *et al.*, 2013; Stephenson *et al.*, 2018). Reducing monocyte infiltration and activation has previously improved clinical scores in EAE mice (Niimi *et al.*, 2013). Moreover, blocking monocyte entry into the CNS in *Ccr2* null mice delayed EAE onset, while enhancing monocyte infiltration via SOCS3 deficiency accelerated disease onset and exacerbated neurological disability in EAE (Saederup *et al.*, 2010; Qin *et al.*, 2012).

Mechanistically, our findings suggest that Nrg-1β1 may suppress monocyte infiltration by its remarkable ability to reduce the activity of CSPGs and MMP9. Recent studies in multiple sclerosis and EAE uncovered that CSPGs facilitate leucocyte accumulation in the perivascular cuff and promote their trafficking into the CNS (Stephenson *et al.*, 2018; Stephenson and Yong, 2018). Our studies in spinal cord injury also identified a pro-inflammatory role for CSPG signalling (Dyck *et al.*, 2018). Leucocyte infiltration also requires activation of MMPs that are expressed by microglia, monocytes and macrophages (Yong *et al.*, 2001; Nuttall *et al.*, 2007; Rawji and Yong, 2013). MMP9, in particular, plays a key role in disruption of the blood–CNS barrier and promoting multiple sclerosis pathogenesis (McManus *et al.*, 1998; Larochelle *et al.*, 2011; Gerwien *et al.*, 2016). As supporting evidence, studies in cortical injury showed that NRG1 treatment reduces injury-induced permeability of endothelial cells in the blood–CNS barrier by attenuating IL-1β (Lok *et al.*, 2012). Of interest, our previous studies in spinal cord injury and LPC-induced focal lesions also showed a reduction in CSPGs production and MMP9 activity in the spinal cord under Nrg-1β1 treatment (Alizadeh *et al.*, 2017; Kataria *et al.*, 2018), suggesting a common immunomodulatory mechanism for Nrg-1β1 in CNS inflammation. CSPGs and MMP9 can be produced by multiple cell types in EAE and other inflammatory conditions including activated astrocytes, microglia and infiltrating monocyte derived macrophages (Asher *et al.*, 2000; Properzi *et al.*, 2005; Silver and Silver, 2014; Dyck and Karimi-Abdolrezaee, 2015; Hallmann *et al.*, 2015; Stephenson *et al.*, 2018). Moreover, as shown in our study and previous reports, these cells express Nrg-1β1 binding receptor ERBB2 and ErRBB4 under normal and inflammatory conditions (Calvo *et al.*, 2010; Alizadeh *et al.*, 2017; Chen *et al.*, 2017; Schumacher *et al.*, 2017; Shahriary *et al.*, 2019). Thus, Nrg-1β1 could potentially attenuates the production of MMP9 or CSPGs directly by influencing activated astrocytes, microglia and macrophages in EAE. However, further studies with cell-specific targeted approaches are warranted to dissect the role of Nrg-1β1 in regulating the expression of CSPGs and MMP9 under inflammatory microenvironment.

Interestingly, Nrg-1β1 did not influence microglia recruitment into EAE lesions, while it fostered a phenotype shift in $CD11b^+$ microglia and macrophages towards anti-inflammatory M2-like phenotype with a concomitant decrease in pro-inflammatory M1-like cells. This is a desirable therapeutic outcome in EAE and multiple sclerosis, as microglia and macrophages are also critical in facilitating remission in multiple sclerosis (Miron *et al.*, 2013). Heterogeneity of microglia and their diverse activated phenotype is increasingly recognized in multiple sclerosis pathophysiology (Plemel *et al.*, 2020). Recent work showed that microglia even attenuate the toxic effects of macrophages in demyelinating lesions (Plemel *et al.*, 2020). Depletion of M2-like microglia and macrophages has impaired remyelination (Olah *et al.*, 2012; Miron *et al.*, 2013), and our previous *in vitro* studies also identified that availability of Nrg-1β1 can restore the suppressed phagocytic properties of pro-inflammatory microglia (Shahriary *et al.*, 2019), which is a prerequisite for successful repair and remyelination. Collectively, our findings support a positive role for Nrg-1β1 in fostering a reparative phenotype in microglia. The positive effects of Nrg-1β1 on microglia phenotype may explain the results of previous studies by our group and others in rodent models of CNS injury and demyelination that showed NRG1 promotes endogenous oligodendrogenesis, preserves axons and promotes spontaneous remyelination (Cannella *et al.*, 1998; Gauthier *et al.*, 2013; Alizadeh *et al.*, 2017; Kataria *et al.*, 2018). However, further studies are needed to elucidate the specific effects of Nrg-1β1 on neurons, axons, and oligodendrocytes in the context of EAE.

We demonstrate that Nrg-1β1 specifically regulated $IFN\gamma^+$ Th1 effector cells in EAE mice without any apparent role in Th17 response. Interestingly, unlike monocytes, Nrg-1β1 regulation of Th1 response takes place at the CNS levels and not peripherally. Based on our *in vitro* study, Nrg-1β1 influenced Th1 polarization directly and indirectly through modulation of macrophages. These findings are supported by our previous study, in which systemic Nrg-1β1 suppressed $IFN\gamma^+$ effector T cells in traumatic spinal cord injury in rats (Alizadeh *et al.*, 2018). IFNγ is implicated in the pathogenesis of multiple sclerosis and EAE (Zamvil and Steinman, 1990) and its intrathecal administration promotes early disease onset in EAE (Furlan *et al.*, 2001). The effects of IFNγ on APCs are pleiotropic and encompass upregulation of major histocompatibility complex molecules, induction of reactive oxygen species, phagocytic activity, and increased production of pro-inflammatory cytokines. In fact, EAE is dependent on IFNγ induced production of MCP1 (CCL2) and CXCL10 that facilitate monocytes infiltration into the white matter (Wen *et al.*, 2010). Thus, reduction of $IFN\gamma^+$ Th1 effector cell population and downregulation of MCP1 and CXCL10 chemokines appear to be an underlying mechanism by which Nrg-1β1 regulated EAE progression and recovery in our studies.

Nrg-1β1 promoted a regulatory T-cell response in EAE. Regulatory T cells are known to suppress proliferation and activation of effector T cells by inhibiting autoreactive T cells (Dombrowski *et al.*, 2017; Jones and Hawiger, 2017). Importantly, regulatory T cells mediate recovery from EAE

by attenuating the cytokine production, proliferation and motility of effector T cells in the CNS (Koutrolos *et al.*, 2014). These reports support our findings in this study, where Nrg-1β1-induced increase in the regulatory T-cell population was accompanied by reduced Th1 cells and diminished pro-inflammatory cytokine production in the EAE mice. We previously observed that Nrg-1β1 promotes upregulation of regulatory cytokine IL-10 in spinal cord injury and LPC focal demyelination (Alizadeh *et al.*, 2018; Kataria *et al.*, 2018). However, intriguingly, we did not detect any changes in IL-10 expression in this study indicating an IL-10 independent immunomodulatory mechanism of Nrg-1β1 in our EAE model. Interestingly, a missense mutation in the *NRG1* gene has been associated with immune dysregulation in schizophrenia (Marballi *et al.*, 2010). Individuals carrying the mutation showed significantly elevated levels of IL-1β, IL-6, IL-10, and TNF-α in plasma (Marballi *et al.*, 2010), demonstrating a direct association between dysregulation of NRG1 and immune cell overactivation and cytokine production. Taken together, based on our new findings in EAE, we propose that endogenous Nrg-1β1 is important for immune homeostasis and its dysregulation is a disease mechanism that facilitates EAE onset and progression by promoting monocyte extravasation and inducing an IFNγ Th1 response. To address this hypothesis, future conditional knockout studies are required to elucidate whether the absence of Nrg-1β1 would result in immune dysregulation peripherally and in the CNS.

A major disadvantage of available immunosuppressive therapies in multiple sclerosis is that they generally impair T-cell functions that can adversely increase the risk for systemic infections and comorbidities (Mills and Mao-Draayer, 2018). Identifying specific immune regulatory mechanisms of multiple sclerosis pathology would allow development of targeted treatments. Our work has identified an endogenous pathway that appears to play a role in immune homeostasis, and that its disruption is associated with multiple sclerosis pathogenesis. In addition to its potential as an early disease marker, Nrg-1β1 represents a desirable specific immune regulatory therapy, as its restoration can moderate the imbalanced immune response and disease severity in EAE. We have identified several potential therapeutic advantages of Nrg-1β1 treatment for multiple sclerosis. First, this treatment is aimed to restore the dysregulated levels of endogenous Nrg-1β1 and not over-activating a pathway that may result in adverse effects. Second, Nrg-1β regulates the phenotype of innate and adaptive immune cells rather than suppressing the immune response. Third, and intriguingly, Nrg-1β1 treatment offers an extended therapeutic time window at least in EAE mice by showing efficacy when administered at various points during the course of the disease. Lastly, an important property of Nrg-1β1 peptide is its desirable pharmacokinetics for CNS therapeutics, as its ability to pass the blood–CNS barrier is confirmed (Kastin *et al.*, 2004). Accordingly, Nrg-1β1 encompasses several characteristics that makes it a potential therapeutic candidate for further investigations in multiple sclerosis.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

References

Agrawal SM, Silva C, Tourtellotte WW, Yong VW. EMMPRIN: a novel regulator of leukocyte transmigration into the CNS in multiple sclerosis and experimental autoimmune encephalomyelitis. J Neurosci 2011; 31: 669–77.

Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci 2011; 14: 1142–9.

Alizadeh A, Dyck SM, Kataria H, Shahriary GM, Nguyen DH, Santhosh KT, et al. Neuregulin-1 positively modulates glial response and improves neurological recovery following traumatic spinal cord injury. Glia 2017; 65: 1152–75.

Alizadeh A, Santhosh KT, Kataria H, Gounni AS, Karimi-Abdolrezaee S. Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury. J Neuroinflammation 2018; 15: 53.

Asher RA, Morgenstern DA, Fidler PS, Adcock KH, Oohira A, Braistead JE, et al. Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. J Neurosci 2000; 20: 2427–38.

Baaklini CS, Rawji KS, Duncan GJ, Ho MFS, Plemel JR. Central nervous system remyelination: roles of Glia and innate immune cells. Front Mol Neurosci 2019; 12: 225.

Bahadori Z, Behmanesh M, Sahraian MA. Two functional promoter polymorphisms of neuregulin 1 gene are associated with progressive forms of multiple sclerosis. J Neurol Sci 2015; 351: 154–9.

Bartus K, Galino J, James ND, Hernandez-Miranda LR, Dawes JM, Fricker FR, et al. Neuregulin-1 controls an endogenous repair mechanism after spinal cord injury. Brain 2016; 139: 1394–416.

Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 2009; 25: 1091–3.

Bizzozero OA, DeJesus G, Callahan K, Pastuszyn A. Elevated protein carbonylation in the brain white matter and gray matter of patients with multiple sclerosis. J Neurosci Res 2005; 81: 687–95.

Brambilla R. The contribution of astrocytes to the neuroinflammatory response in multiple sclerosis and experimental autoimmune encephalomyelitis. Acta Neuropathol 2019; 137: 757–83.

Calvo M, Zhu N, Tsantoulas C, Ma Z, Grist J, Loeb JA, et al. Neuregulin-ErbB signaling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain after peripheral nerve injury. J Neurosci 2010; 30: 5437–50.

Cannella B, Hoban CJ, Gao YL, Garcia-Arenas R, Lawson D, Marchionni M, et al. The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. Proc Natl Acad Sci USA 1998; 95: 10100–5.

Chen J, He W, Hu X, Shen Y, Cao J, Wei Z, et al. A role for ErbB signaling in the induction of reactive astrogliosis. Cell Discov 2017; 3: 17044. doi: 10.1038/celldisc.2017.44.

Choi BY, Kim JH, Kho AR, Kim IY, Lee SH, Lee BE, et al. Inhibition of NADPH oxidase activation reduces EAE-induced white matter damage in mice. J Neuroinflammation 2015; 12: 104.

Dombrowski Y, O'Hagan T, Dittmer M, Penalva R, Mayoral SR, Bankhead P, et al. Regulatory T cells promote myelin regeneration in the central nervous system. Nat Neurosci 2017; 20: 674–80.

Dong Y, Yong VW. When encephalitogenic T cells collaborate with microglia in multiple sclerosis. Nat Rev Neurol 2019; 15: 704–17.

Dyck SM, Karimi-Abdolrezaee S.Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. Exp Neurol 2015; 269: 169.doi: 10.1016/j.expneurol.2015.04.006.

Dyck S, Kataria H, Alizadeh A, Santhosh KT, Lang B, Silver J, et al. Perturbing chondroitin sulfate proteoglycan signaling through LAR and PTPsigma receptors promotes a beneficial inflammatory response following spinal cord injury. J Neuroinflammation 2018; 15: 90.

Faissner S, Plemel JR, Gold R, Yong VW. Progressive multiple sclerosis: from pathophysiology to therapeutic strategies. Nat Rev Drug Discov 2019; 18: 905–22.

Fischer MT, Wimmer I, Hoftberger R, Gerlach S, Haider L, Zrzavy T, et al. Disease-specific molecular events in cortical multiple sclerosis lesions. Brain 2013; 136 (Pt 6): 1799–815.

Furlan R, Brambilla E, Ruffini F, Poliani PL, Bergami A, Marconi PC, et al. Intrathecal delivery of IFN-gamma protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. J Immunol 2001; 167: 1821–9.

Gauthier MK, Kosciuczyk K, Tapley L, Karimi-Abdolrezaee S. Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. Eur J Neurosci 2013; 38: 2693–715.

Gerwien H, Hermann S, Zhang X, Korpos E, Song J, Kopka K, et al. Imaging matrix metalloproteinase activity in multiple sclerosis as a specific marker of leukocyte penetration of the blood-brain barrier. Sci Transl Med 2016; 8: 364ra152.

Hallmann R, Zhang X, Di Russo J, Li L, Song J, Hannocks MJ, et al. The regulation of immune cell trafficking by the extracellular matrix. Curr Opin Cell Biol 2015; 36: 54–61.

Ikawa D, Makinodan M, Iwata K, Ohgidani M, Kato TA, Yamashita Y, et al. Microglia-derived neuregulin expression in psychiatric disorders. Brain Behav Immun 2017; 61: 375–85.

Jiang Z, Jiang JX, Zhang GX. Macrophages: a double-edged sword in experimental autoimmune encephalomyelitis. Immunol Lett 2014; 160: 17–22.

Jones A, Hawiger D. Peripherally induced regulatory T cells: recruited protectors of the central nervous system against autoimmune neuroinflammation. Front Immunol 2017; 8: 532.

Kastin AJ, Akerstrom V, Pan W. Neuregulin-1-beta1 enters brain and spinal cord by receptor-mediated transport. J Neurochem 2004; 88: 965–70.

Kataria H, Alizadeh A, Karimi-Abdolrezaee S. Neuregulin-1/ErbB network: an emerging modulator of nervous system injury and repair. Prog Neurobiol 2019; 180: 101643.

Kataria H, Alizadeh A, Shahriary GM, Saboktakin Rizi S, Henrie R, Santhosh KT, et al. Neuregulin-1 promotes remyelination and fosters a pro-regenerative inflammatory response in focal demyelinating lesions of the spinal cord. Glia 2018; 66: 538–61.

Koutrolos M, Berer K, Kawakami N, Wekerle H, Krishnamoorthy G. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. Acta Neuropathol Commun 2014; 2: 163.

Kuhle J, Disanto G, Dobson R, Adiutori R, Bianchi L, Topping J, et al. Conversion from clinically isolated syndrome to multiple sclerosis: a large multicentre study. Mult Scler 2015; 21: 1013–24.

Larochelle C, Alvarez JI, Prat A. How do immune cells overcome the blood-brain barrier in multiple sclerosis? FEBS Lett 2011; 585: 3770–80.

Li Q, Zhang R, Guo YL, Mei YW. Effect of neuregulin on apoptosis and expressions of STAT3 and GFAP in rats following cerebral ischemic reperfusion. J Mol Neurosci 2009; 37: 67–73.

Li Y, Lein PJ, Ford GD, Liu C, Stovall KC, White TE, et al. Neuregulin-1 inhibits neuroinflammatory responses in a rat model of organophosphate-nerve agent-induced delayed neuronal injury. J Neuroinflammation 2015; 12: 64.

Loeb JA, Khurana TS, Robbins JT, Yee AG, Fischbach GD. Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. Development 1999; 126: 781–91.

Loeb JA, Susanto ET, Fischbach GD. The neuregulin precursor proARIA is processed to ARIA after expression on the cell surface by a protein kinase C-enhanced mechanism. Mol Cell Neurosci 1998; 11: 77–91.

Lok J, Zhao S, Leung W, Seo JH, Navaratna D, Wang X, et al. Neuregulin-1 effects on endothelial and blood-brain-barrier permeability after experimental injury. Transl Stroke Res 2012; 3 (Suppl 1): S119–24.

Marballi K, Quinones MP, Jimenez F, Escamilla MA, Raventos H, Soto-Bernardini MC, et al. In vivo and in vitro genetic evidence of involvement of neuregulin 1 in immune system dysregulation. J Mol Med 2010; 88: 1133–41.

McMahon EJ, Bailey SL, Castenada CV, Waldner H, Miller SD. Epitope spreading initiates in the CNS in two mouse models of multiple sclerosis. Nat Med 2005; 11: 335–9.

McManus C, Berman JW, Brett FM, Staunton H, Farrell M, Brosnan CF. MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study. J Neuroimmunol 1998; 86: 20–9.

Mei L, Nave KA. Neuregulin-ERBB signaling in the nervous system and neuropsychiatric diseases. Neuron 2014; 83: 27–49.

Mei L, Xiong WC. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nat Rev Neurosci 2008; 9: 437–52.

Metz LM, Li DKB, Traboulsee AL, Duquette P, Eliasziw M, Cerchiaro G, et al. Trial of minocycline in a clinically isolated syndrome of multiple sclerosis. N Engl J Med 2017; 376: 2122–33.

Michailov GV, Sereda MW, Brinkmann BG, Fischer TM, Haug B, Birchmeier C, et al. Axonal neuregulin-1 regulates myelin sheath thickness. Science 2004; 304: 700–3.

Miller DH, Chard DT, Ciccarelli O. Clinically isolated syndromes. Lancet Neurol 2012; 11: 157–69.

Mills EA, Mao-Draayer Y. Understanding progressive multifocal leukoencephalopathy risk in multiple sclerosis patients treated with immunomodulatory therapies: a bird's eye view. Front Immunol 2018; 9: 138.

Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci 2013; 16: 1211–8.

Moline-Velazquez V, Vila-Del Sol V, de Castro F, Clemente D. Myeloid cell distribution and activity in multiple sclerosis. Histol Histopathol 2016; 31: 357–70.

Nave KA, Salzer JL. Axonal regulation of myelination by neuregulin 1. Curr Opin Neurobiol 2006; 16: 492–500.

Niimi N, Kohyama K, Matsumoto Y. Minocycline suppresses experimental autoimmune encephalomyelitis by increasing tissue inhibitors of metalloproteinases. Neuropathology 2013; 33: 612–20.

Nikic I, Merkler D, Sorbara C, Brinkoetter M, Kreutzfeldt M, Bareyre FM, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat Med 2011; 17: 495–9.

Nuttall RK, Silva C, Hader W, Bar-Or A, Patel KD, Edwards DR, et al. Metalloproteinases are enriched in microglia compared with leukocytes and they regulate cytokine levels in activated microglia. Glia 2007; 55: 516–26.

Olah M, Amor S, Brouwer N, Vinet J, Eggen B, Biber K, et al. Identification of a microglia phenotype supportive of remyelination. Glia 2012; 60: 306–21.

Pankonin MS, Sohi J, Kamholz J, Loeb JA. Differential distribution of neuregulin in human brain and spinal fluid. Brain Res 2009; 1258: 1–11.

Plemel JR, Stratton JA, Michaels NJ, Rawji KS, Zhang E, Sinha S, et al. Microglia response following acute demyelination is heterogeneous and limits infiltrating macrophage dispersion. Sci Adv 2020; 6: eaay6324.

Properzi F, Carulli D, Asher RA, Muir E, Camargo LM, van Kuppevelt TH, et al. Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia. Eur J Neurosci 2005; 21: 378–90.

Qin H, Holdbrooks AT, Liu Y, Reynolds SL, Yanagisawa LL, Benveniste EN. SOCS3 deficiency promotes M1 macrophage polarization and inflammation. Ji 2012; 189: 3439–48.

Rawji KS, Yong VW. The benefits and detriments of macrophages/microglia in models of multiple sclerosis. Clin Dev Immunol 2013; 2013: 948976.

Reich DS, Lucchinetti CF, Calabresi PA. Multiple sclerosis. N Engl J Med 2018; 378: 169–80.

Rostami A, Ciric B. Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. J Neurol Sci 2013; 333: 76–87.

Saederup N, Cardona AE, Croft K, Mizutani M, Cotleur AC, Tsou CL, et al. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. PLoS One 2010; 5: e13693.

Schumacher MA, Hedl M, Abraham C, Bernard JK, Lozano PR, Hsieh JJ, et al. ErbB4 signaling stimulates pro-inflammatory macrophage apoptosis and limits colonic inflammation. Cell Death Dis 2017; 8: e2622. doi: 10.1038/cddis.2017.42.

Shahriary GM, Kataria H, Karimi-Abdolrezaee S. Neuregulin-1 fosters supportive interactions between microglia and neural stem/progenitor cells. Stem Cells Int 2019; 2019: 8397158.

Silver DJ, Silver J. Contributions of chondroitin sulfate proteoglycans to neurodevelopment, injury, and cancer. Curr Opin Neurobiol 2014; 27: 171–8. doi: 10.1016/j.conb.2014.03.016.

Simmons LJ, Surles-Zeigler MC, Li Y, Ford GD, Newman GD, Ford BD. Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway. J Neuroinflammation 2016; 13: 237.

Skundric DS, Cruikshank WW, Drulovic J. Role of IL-16 in CD4+ T cell-mediated regulation of relapsing multiple sclerosis. J Neuroinflammation 2015; 12: 78.

Sosa RA, Murphey C, Ji N, Cardona AE, Forsthuber TG. The kinetics of myelin antigen uptake by myeloid cells in the central nervous system during experimental autoimmune encephalomyelitis. J Immunol 2013; 191: 5848–57.

Stephenson EL, Mishra MK, Moussienko D, Laflamme N, Rivest S, Ling CC, et al. Chondroitin sulfate proteoglycans as novel drivers of leucocyte infiltration in multiple sclerosis. Brain 2018; 141: 1094–110.

Stephenson EL, Yong VW. Pro-inflammatory roles of chondroitin sulfate proteoglycans in disorders of the central nervous system. Matrix Biol 2018; 71-72: 432–42.

Stephenson EL, Zhang P, Ghorbani S, Wang A, Gu J, Keough MB, et al. Targeting the chondroitin sulfate proteoglycans: evaluating fluorinated glucosamines and xylosides in screens pertinent to multiple sclerosis. ACS Cent Sci 2019; 5: 1223–34.

Tokita Y, Keino H, Matsui F, Aono S, Ishiguro H, Higashiyama S, et al. Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. J Neurosci 2001; 21: 1257–64.

Tynyakov-Samra E, Auriel E, Levy-Amir Y, Karni A. Reduced ErbB4 expression in immune cells of patients with relapsing remitting multiple sclerosis. Mult Scler Int 2011; 2011: 561262.

van Zwam M, Samsom JN, Nieuwenhuis EE, Melief MJ, Wierenga-Wolf AF, Dijke IE, et al. Myelin ingestion alters macrophage antigen-presenting function in vitro and in vivo. J Leukoc Biol 2011; 90: 123–32.

Viehover A, Miller RH, Park SK, Fischbach G, Vartanian T. Neuregulin: an oligodendrocyte growth factor absent in active multiple sclerosis lesions. Dev Neurosci 2001; 23: 377–86.

Weinshenker BG, Bass B, Rice GP, Noseworthy J, Carriere W, Baskerville J, et al. The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. Brain 1989; 112 (Pt 1): 133–46.

Wen L, Lu YS, Zhu XH, Li XM, Woo RS, Chen YJ, et al. Neuregulin 1 regulates pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons. Proc Natl Acad Sci USA 2010; 107: 1211–6.

Xu Z, Croslan DR, Harris AE, Ford GD, Ford BD. Extended therapeutic window and functional recovery after intraarterial administration of neuregulin-1 after focal ischemic stroke. J Cereb Blood Flow Metab 2006; 26: 527–35.

Yong HYF, Rawji KS, Ghorbani S, Xue M, Yong VW. The benefits of neuroinflammation for the repair of the injured central nervous system. Cell Mol Immunol 2019; 16: 540–6.

Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2001; 2: 502–11.

Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. Annu Rev Immunol 1990; 8: 579–621.